UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA *TUISKON DICK*

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

EFEITO DO GLICOLALDEÍDO SOBRE PARÂMETROS DE ESTRESSE OXIDATIVO NO RIM, FÍGADO E CORAÇÃO DE RATOS WISTAR

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PORTO ALEGRE, AGOSTO DE 2010.

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“Everybody’s looking for the Sun

People strain their eyes to see

But I see you and you see me

Ain’t that wonder?”

Ray Davies

SUMÁRIO

RESUMO

O glicolaldeído é um aldeído hidroxilado de dois carbonos capaz de reagir com proteínas, alterando sua estrutura e função. Ele é formado como subproduto do metabolismo glicolítico, da reação da glicose com proteínas e também a partir da ação da mieloperoxidase sobre aminoácidos. Logo, situações de hiperglicemia e inflamação favorecem a formação do glicolaldeído. Sua reação com biomoléculas, além de prejudicar a função das mesmas, pode levar à produção de produtos finais de glicação avançada (AGEs). Diversos estudos têm relacionado o acúmulo de AGEs com diversas doenças como *diabetes mellitus*, mal de Alzheimer, falência renal e o próprio envelhecimento. Os AGEs apresentam boa parte de seus efeitos deletérios devidos à sua ligação com o receptor RAGE. Sabe-se que este mecanismo, de forma geral, promove uma resposta inflamatória, gerando estresse oxidativo. Entretanto, apesar dos grandes avanços no estudo dos AGEs e seu efeitos, muito pouco se sabe sobre a ação direta de precursores dos AGEs, como o glicolaldeído. Os objetivos deste trabalho foram investigar os efeitos agudos do glicolaldeído sobre parâmetros de estresse oxidativo no rim, coração e fígado de ratos. Ratos Wistar machos adultos receberam 10, 50 ou 100 mg/Kg de glicolaldeído através de injeção intravenosa. Os animais foram sacrificados 6, 12 e 24 horas após a injeção. Nós observamos um aumento dos marcadores de estresse oxidativo (carbonilação proteica, lipoperoxidação e diminuição de tióis reduzidos) em todas as estruturas analisadas. As enzimas superóxido dismutase, catalase e glioxalase I tiveram suas atividades moduladas pela injeção do glicolaldeído nos três órgãos estudados. Além disso a injeção do aldeído provocou o acúmulo de carboximetil-lisina, um dos AGEs mais estudados, no fígado dos animais. Nossos resultados sugerem que mesmo curtos eventos de hiperglicemia e inflamação, capazes de aumentar os níveis de glicolaldeído, podem gerar estresse oxidativo e, de forma cumulativa, favorecer as complicações observadas em doenças como o *diabetes mellitus*.

ABSTRACT

Glycolaldehyde is a two-carbon aldehyde that reacts with proteins, modifying their structure and impairing their function. It is formed as a byproduct of the non-enzymatic reaction between glucose and biomolecules and also from the action of myeloperoxidase upon amino acids. Thus, events of hyperglycemia and inflammation favor the formation of glycolaldehyde. Despite impairing protein function, glycolaldehyde might lead to the formation of advanced glycation end-products (AGEs). There are several studies relating AGEs to many diseases such as *diabetes mellitus*, Alzheimer's disease, renal failure and the aging process itself. Part of the deleterious effects of AGEs is due to interaction with their receptor (RAGE). There is evidence that this interaction induces inflammatory responses and oxidative stress. Despite the huge advances in understanding the effects of AGEs, there are very few reports describing the effects of precursors of AGEs, such as glycolaldehyde. The objectives of this work were to investigate the acute effects of glycolaldehyde on oxidative stress parameters in the kidney, heart and liver of rats. Male adult Wistar rats received a single intravenous injection of glycolaldehyde at 10, 50 or 100 mg/Kg. The animals were sacrificed 6, 12 or 24 hours after injection. We observed an increase in markers of oxidative stress (protein carbonylation, lipoperoxidation and a decrease in reduced thiol content). The activities of superoxide dismutase, catalase and glyoxalase I were modulated by the injection of glycolaldehyde. Moreover, glycolaldehyde induced accumulation of $N^{\pounds}$-carboxymethyl(lysine), one of the most studied AGEs. Our results suggest that even short-term events of hyperglycemia and inflammation, capable of raising glycolaldehyde levels, might generate oxidative stress and, in a cumulative way, favor the onset of complications such as those observed in *diabetes mellitus*.

LISTA DE ABREVIATURAS

AGEs - produtos finais de glicação avançada

CAT - catalase

CEL - carboxietil-lisina

CMC - carboximetilcisteína

CML - carboximetil-lisina

DM - *diabetes mellitus*

ERO - espécies reativas de oxigênio

GA - glicolaldeído

GLO - glioxalase I

GPx - glutationa peroxidase

GSH - glutationa reduzida

H_2O_2 - peróxido de hidrogênio

HMGB1 – Proteína do grupo de alta mobilidade 1

$O_2^{\bullet-}$ - ânion superóxido

$OH^{\bullet}$ - radical hidroxil

LDL - lipoproteína de baixa densidade

MG - metilglioxal

NF-κB – Fator nuclear κB

$NO^{\bullet}$ - óxido nítrico

RAGE - receptor de produtos finais de glicação avançada

SOD - superóxido dismutase

1. INTRODUÇÃO

1.1 Glicolaldeído e Produtos Finais de Glicação Avançada

O glicolaldeído (GA) é um aldeído de dois carbonos formado como subproduto da glicólise (Glomb & Monnier, 1995) e da atividade da mieloperoxidase de neutrófilos sobre aminoácidos (Anderson *et al.*, 1997). Logo, hiperglicemia e inflamação, características de pacientes diabéticos, favorecem sua formação. O GA é bastante reativo com resíduos de lisina e arginina. O que ocorre nesses casos é a reação do grupamento carbonil (C=O) do GA com a porção amino lateral destes aminoácidos. Sabe-se também que o GA reage com resíduos de cisteína. Tanto a reação com lisina e arginina quanto a reação com cisteína podem levar à formação de produtos finais de glicação avançada (AGEs, do inglês Advanced Glycation End-products). Entretanto, os mecanismos que levam à formação dos AGEs diferem nos dois casos. Ao reagir com a cisteína, o GA forma um tiohemiacetal que sofre rearranjo, gerando o AGE carboximetilcisteína (CMC). Reagindo com lisina ou arginina, forma-se uma base de Schiff que sofre rearranjos, gerando estáveis produtos de Amadori e então, AGEs.

Devido à sua alta reatividade e à estabilidade dos produtos formados, o GA pode modificar a estrutura de biomoléculas, alterando sua função e comprometendo o funcionamento celular. Já foi demonstrado que o GA modifica albumina, reduzindo sua capacidade de ligação às drogas varfarina e cetoprofeno (Mera *et al.*, 2010). Nosso grupo já demonstrou que o GA modifica estruturalmente o fibrinogênio, promovendo um atraso no tempo de formação do coágulo, bem como uma maior resistência à degradação enzimática (Andrades *et al.*, 2009). Esse dado em especial

corrobora dados da literatura que associam hiperglicemia e inflamação a quadros pró-trombóticos. De fato, o diabetes mellitus (DM), caracterizado pela ineficiência na regulação dos níveis de glicose circulante, apresenta como principal causa de mortalidade as complicações cardiovasculares. A glicação de lipoproteínas de baixa densidade (LDL) pelo GA favorece a captação de colesterol e éster de colesterol por macrófagos, facilitando a formação de células espumosas (Brown, Dean & Davies, 2005). Estas células contribuem para a formação de placas ateroscleróticas. È interessante ressaltar que outros aldeídos com mecanismo de ação parecido com o GA também são formados em situações patológicas. Metilglioxal (MG) e glioxal são os mais estudados. Ambos são capazes de alterar a albumina e reduzir sua capacidade ligante, sendo o MG mais efetivo neste parâmetro (Mera *et al.*, 2010). Os três aldeídos diferem também na especificidade de reação com aminoácidos e nos AGEs formados. Glioxal e MG reagem preferencialmente com resíduos de arginina, enquanto o GA tem maior afinidade por resíduos de lisina. Além disso, o próprio GA, num processo de enolização, pode gerar glioxal (Al-Enezi, Alkhalaf & Benov, 2006). Os AGEs derivados do GA são carboximetil-lisina (CML) e GA-piridina. O glioxal gera CML e imidazolona, enquanto o MG forma principalmente carboxietil-lisina (CEL). A figura 1 mostra um mecanismo generalizado de formação de AGEs.

1.2 Efeitos toxicológicos dos AGEs

Os AGEs são estruturas estáveis e tendem a se acumular no organismo durante a vida. Entretanto, esta acumulação é acentuada em doenças como o DM.

Diabéticos apresentam maiores níveis de AGEs no sangue, em comparação com indivíduos saudáveis. O nível de glicação do colágeno da pele é considerado um marcador para complicações microvasculares, tais como a nefropatia diabética (Genuth *et al.*, 2005; Gerrits *et al.*, 2008). A acumulação de AGEs também está relacionada com o desenvolvimento da falência renal (Beisswenger *et al.*, 1995). Camundongos tratados com albumina glicada apresentam resistência à insulina. Essa resistência promove aumento nos níveis de glicose durante o jejum (Cassese *et al.*, 2008). Os AGEs também reduzem a secreção de insulina pelas células beta do pâncreas, além de acumular neste órgão (Tajiri, Moller & Grill, 1997). Estes trabalhos evidenciam a importância dos AGEs no desenvolvimento da condição diabética.

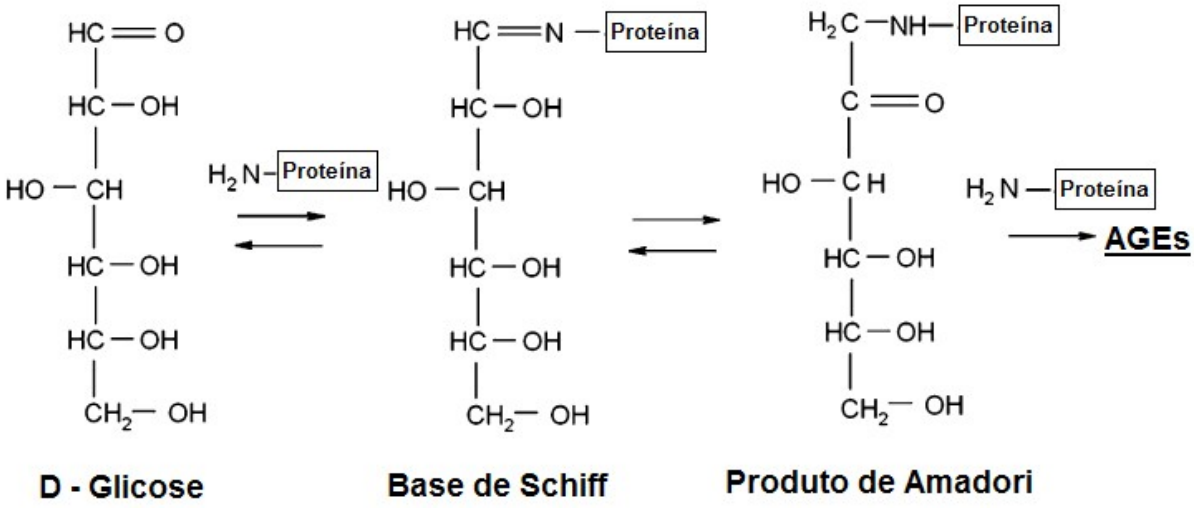

Figura 1. Reações iniciais da formação de produtos finais de glicação avançada (AGEs) a partir de glicose. Retirado de Grillo e Colombatto (Grillo & Colombatto, 2008).

Além de constituir uma alteração estrutural *per se*, os AGEs também podem desencadear efeitos celulares através da ligação com seu receptor (RAGE). O RAGE é um receptor de membrana com um domínio externo constituído de três regiões *imunoglobulina-like*, além de um domínio transmembrana com projeção para

o citosol (Chavakis, Bierhaus & Nawroth, 2004). Além dos AGEs, proteínas inflamatórias como a S100β e HMGB1 também são reconhecidas pelo RAGE. A ligação com o RAGE ativa uma gama de rotas de sinalização, passando por MAP cinases, fosfoinositol-3 cinase e também pela ativação de NFκB. De fato, em modelo animal de sepse, a injeção de albumina modificada com CML aumenta a mortalidade dos animais, num mecanismo dependente da ativação de NFκB. Em animais que não expressam RAGE, a mortalidade é diminuída (Humpert *et al.*, 2009).

O RAGE parece estar envolvido em eventos de estresse oxidativo. Em camundongos diabéticos, a deficiência de RAGE reduz a produção mitocondrial do radical ânion superóxido, além de reduzir os níveis de apoptose (Coughlan *et al.*, 2009). Células progenitoras endoteliais, responsáveis pela reparação do endotélio, apresentam um aumento na produção de espécies reativas de oxigênio (ERO), além de reduzirem a expressão de enzimas antioxidantes. A inibição do RAGE por RNA de interferência é capaz de reverter estes efeitos (Chen *et al.*, 2010). Evidências sugerem que o bloqueio da interação entre AGEs e RAGE tem um promissor potencial terapêutico em doenças cardiovasculares, DM e mal de Alzheimer (Yamagishi, Nakamura & Matsui, 2009; D'agati & Schmidt, 2010; Fang *et al.*, 2010).

1.3 Radicais livres e estresse oxidativo

Um radical livre é qualquer espécie química que possua um ou mais elétrons desemparelhados em seu ultimo orbital. Tais espécies podem ser átomos ou moléculas. Estes átomos ou moléculas apresentam alta reatividade devido à grande tendência em adquirir um segundo elétron para o orbital (Halliwell, 2006). Radicais

livres são escritos quimicamente com uma notação para a espécie química seguida de um ponto, o qual indica o elétron desemparelhado, por exemplo, o radical livre ânion superóxido: $O_2^{\bullet-}$.

Classicamente, as reações de radicais livres são divididas em: a) reações de iniciação; b) reações de propagação; e c) reações de terminação. Nas reações de iniciação, um radical livre é formado a partir de espécies químicas não-radicais: AB + C → $A^{\bullet}$ + D + E. Nas reações de propagação, um radical livre, também chamado centro de reação, reage com uma molécula estável, resultando em outro radical livre ou centro de reação: $A^{\bullet}$ + CD → AC + $D^{\bullet}$. Nas reações de terminação, dois radicais livres cancelam seus elétrons desemparelhados formando um produto estável.

O radical livre que ocorre mais comumente em sistemas biológicos é o ânion superóxido ($O_2^{\bullet-}$), que é produzido quando uma molécula de oxigênio é reduzida parcialmente, ou seja, quando recebe apenas um elétron. Quando em excesso, o radical superóxido pode levar à produção de peróxido de hidrogênio (H_2O_2), através da atividade da enzima superóxido dismutase (SOD):

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

O peróxido formado, reagindo com metais de transição como Fe^{+2} e Cu^{+2}, pode gerar o radical hidroxil ($HO^{\bullet}$), em uma reação denominada reação de Fenton. Outro destino do superóxido é a reação com óxido nítrico ($NO^{\bullet}$), formando o peroxinitrito ($ONOO^{\bullet}$) que, por sua vez, pode gerar o nitrosil (HONOO). Este, por sua vez, sofrendo decomposição, também forma o radical hidroxil.

O estresse oxidativo é caracterizado por um desequilíbrio entre a produção de espécies reativas e as defesas antioxidantes, em favor do primeiro, podendo gerar dano (Halliwell, 2007). As defesas antioxidantes podem ser enzimáticas ou não-enzimáticas. Entre as defesas não-enzimáticas podemos citar o tripeptídeo

glutationa (GSH, na forma reduzida) e as vitaminas como o ácido ascórbico e a vitamina E. Dentre as defesas enzimáticas, as mais conhecidas são a SOD, a catalase (CAT) e a glutationa peroxidase (GPx). A catalase decompõe o peróxido de hidrogênio, gerando água e oxigênio:

$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

A enzima GPx também decompõe o peróxido, porém, fazendo uso de um mecanismo diferente. Ela utiliza a GSH para transformar o H_2O_2 em água. A glutationa oxidada nesta reação é reduzida novamente pela ação da enzima glutationa redutase, consumindo NADPH (Boveris, 1998).

1.4 Estresse oxidativo e doenças humanas

Inúmeros trabalhos descrevem a participação de estresse oxidativo em doenças humanas, apesar da relação causa e efeito não ser estabelecida. De uma forma geral, doenças que apresentam eventos de isquemia/reperfusão e inflamação têm como componente o estresse oxidativo (Giustarini *et al.*, 2009). Estratégias terapêuticas que incluem antioxidantes são alvo constante de pesquisa, embora os avanços efetivos para aplicação de novas terapias não seja tão evidente. Isso se deve principalmente à falta de especificidade dos tratamentos e à imprecisa delimitação do papel do estresse oxidativo nas patologias. Definir os casos onde o desequilíbrio redox é causa ou consequência é uma tarefa bastante delicada e de difícil execução.

O próprio processo de envelhecimento, inerente aos seres vivos, tem como importante componente o estresse oxidativo. Diversos estudos apresentam uma

correlação positiva entre idade e dano oxidativo, além de uma redução do dano em animais tratados para apresentar um aumento no tempo de vida. Apesar da teoria que relaciona envelhecimento e estresse oxidativo estar bem fundamentada no que diz respeito a correlações, experimentos onde há deleção de enzimas antioxidantes são controversos quanto à influência no tempo de vida dos animais (Perez *et al.*, 2009). Este fato reforça a ideia de que a participação de ERO em processos deletérios é um processo multifatorial.

No caso do DM, além da predisposição genética, torna-se complicado dissociar hiperglicemia e estresse oxidativo. Modelos animais de DM mostram dano oxidativo em órgãos como rim (Kuhad & Chopra, 2009) e coração (Shirpoor *et al.*, 2009). De fato, o tratamento com antioxidante atenua os efeitos do DM no que diz respeito a marcadores de estresse oxidativo e função de órgãos.

2. OBJETIVOS

2.1 Objetivos gerais

Hiperglicemia e inflamação favorecem a formação de AGEs. Estes produtos acumulam com o envelhecimento e isso ocorre de forma mais acentuada em diabéticos. Os AGEs, através de seu receptor, podem levar à formação de espécies reativas de oxigênio (ERO), comprometendo a integridade celular e o funcionamento de órgãos como fígado, rins e coração. Apesar de o papel patofisiológico dos AGEs ser bastante evidenciado, muito pouco se sabe sobre os efeitos causados por precursores de AGEs como a glicose e os aldeídos de cadeia curta como o GA.

Assim posto, neste trabalho nós analisamos os efeitos da injeção intravenosa de GA sobre parâmetros de estresse oxidativo no rim, coração e fígado de ratos Wistar machos adultos.

2.2 Objetivos específicos

Analisamos os efeitos de uma única injeção de GA, nas doses de 10, 50 e 100 mg/Kg, após 6, 12 e 24 horas sobre:

1) Carbonilação de proteínas, lipoperoxidação, estado redox de grupamentos sulfidril no rim, coração e fígado;
2) Modulação da atividade das enzimas superóxido dismutase (SOD), catalase (CAT) e glioxalase I (GLO), nas estruturas mencionadas acima;
3) Quantificação de CML nós órgãos mencionados acima.

3. RESULTADOS

Os resultados desta dissertação estão apresentados na forma de artigos aceitos para publicação ou manuscritos submetidos para publicação.

3.1 Circulating glycolaldehyde induces oxidative damage in the kidney of rats

Aritgo aceito para publicação no periódico *Diabetes Research and Clinical Practice*.

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Circulating glycolaldehyde induces oxidative damage in the kidney of rats

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ABSTRACT

Renal failure is a key pathological issue in diabetic patients. Increased levels of advanced glycation end-products (AGEs) have been associated to diabetic complications, including diabetic nephropathy. Models of AGE-treated animals have been applied to evaluate the effect of such molecules on oxidative parameters involved in the pathogenesis and evolution of diabetes disease. However, little is known about the effect of glycating agents other than glucose. Here we investigate the effect of intravenously administrated glycolaldehyde (GA) on oxidative stress parameters of the kidney. Male Wistar rats received a single injection of GA in different doses (10, 50 or 100 mg/kg) and were sacrificed after 6, 12 or 24 h. Activities of antioxidant enzymes catalase, superoxide dismutase and glyoxalase I were assayed. Damage to proteins and lipids were also assayed. The content of N^{ε}-(carboxymethyl)lysine (CML) was quantified. Glycolaldehyde induced a decrease in the activity of all enzymes studied. Lipoperoxidation and protein carbonylation raised, accompanied by a decrease in sulfhydryl groups. Despite the oxidative stress generated by GA, no change was found in the content of CML, suggesting that accumulation of AGEs in the kidney might occur at later steps in the development of diabetic nephropathy.

1. Introduction

Short-chain aldehydes react with amino groups to form a Schiff base, which rearranges to more stable Amadori products that lead to advanced glycation end products (AGEs) [1]. Formation and accumulation of AGEs are associated to diabetic complications. Plasma levels of N^{ε}-(carboxymethyl)lysine (CML) are increased in diabetic rats in comparison to normal animals [2]. The CML content is also increased in soleus muscle [3] and vasculature [4] of diabetic animals.

CML is known to interact with the receptor for AGEs (RAGE) and activate NF-κB. Injection of CML-albumin enhanced mortality of septic mice in a RAGE/NF-κB dependent manner. RAGE −/− mice are protected from this lethality and inflammation [5].

Glycolaldehyde (GA) is a short-chain aldehyde derived as a by-product of protein glycation and myeloperoxidase activity upon L-serine. It may also be derived directly from glucose or Schiff bases by an oxygen-dependent cleavage mechanism [6]. GA reacts with amino groups forming a Schiff base which rearranges to form more stable Amadori products that lead to

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AGE formation. Pyridine, imidazolone and CML are AGEs derived from GA, among other aldehydes [7]. GA preferentially reacts with arginine, lysine and cysteine residues. Such modifications lead to protein dysfunction [8] and oxidative stress [9]. Despite its importance, GA concentrations in plasma of healthy or diabetic patients have not been quantified yet. Nonetheless, physiological concentration is estimated to range from 0.1 to 1 mM [10–12].

Although mechanisms of protein modification are described for GA and so are many of the effects GA-derived AGEs cause in cell homeostasis, little is known about the influence of GA formed *in vivo* on organs function and oxidative status.

In this study, the effects of intravenously administrated GA on acute parameters of oxidative stress in kidney were investigated, in order to evaluate a possible role of GA on the onset and development of diabetic nephropathy.

2. Materials and methods

2.1. Animals and chemicals

Adult male Wistar rats (280–320 g) were obtained from our own breeding colony. They were caged in groups of five with free access to water and food and were maintained on a 12 h light–dark cycle (lights on at 7 a.m.), at a temperature controlled colony room (23 ± 1 °C). These conditions were maintained constant throughout the experiments. All experimental procedures were performed in accordance with the National Institute of Health Guides for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior recommendations for animal care.

All chemicals were purchased from Sigma (St. Louis, USA).

2.2. Treatments

Animals were anesthetized (ketamin 100 mg/kg and xylazin 10 mg/kg) and treated with a single injection of GA via the dorsal vein of the penis, in different doses (10, 50 and 100 mg/kg) in a volume range of 120–150 μL. Control group received 130 μL of NaCl 0.9%.

2.3. Oxidative stress and antioxidant enzymes analysis

Animals were sacrificed at 6, 12 or 24 h after injection. Blood was collected and plasma separated. The kidney was dissected out in ice and immediately stored at −80 °C for posterior analysis. Homogenates were centrifuged ($1000 \times g$, 10 min at 4 °C) to remove cellular debris. Supernatants were used to all biochemical assays described herein. Samples for ELISA were centrifuged one more time ($10{,}000 \times g$, 10 min at 4 °C).

2.3.1. Measurement of protein carbonyl

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with 2,4-dinitrophenylhydrazine (DNPH). Proteins were precipitated by the addition of 20% TCA and ressuspended in 10 mM DNPH and the absorbance read at 370 nm [13]. Results are expressed as nmol carbonyl/mg protein.

2.3.2. Thiobarbituric acid reactive species (TBARS)

As an index of lipoperoxidation we detected thiobarbituric acid reactive species (TBARS) formation through a hot and acidic reaction. This is widely adopted as a method for measurement of lipid redox state, as previously described [14]. Briefly, the samples were mixed with 0.6 mL of 10% trichloroacetic acid (TCA) and 0.5 mL of 0.67% thiobarbituric acid and then heated in a boiling water bath for 25 min. TBARS were determined by absorbance in a spectrophotometer at 532 nm. We have obtained TBARS concentration in the samples from a calibration curve that was performed using 1,1,3,3-tetramethoxypropane as standard, which was subjected to the same treatment as that applied to the supernatants of the samples. Results are expressed as nanomoles TBARS per milligram protein.

2.3.3. Measurement of total reduced thiol content

To quantify the content of reduced thiol, samples were diluted in 10 mM phosphate buffer (pH 7.4) and 0.01 M 5,5′-dithionitrobis 2-nitrobenzoic acid (DTNB) in ethanol was added and the intense yellow color was developed and read at 412 nm after 20 min. A blank sample was run simultaneously, except for the absence of DTNB. Protein thiol content was calculated after subtraction of the blank absorbance from the absorbance of samples with DTNB, utilizing the molar extinction coefficient of 13,600 M^{-1} cm^{-1} [15].

2.3.4. Antioxidant enzyme activity

Catalase (CAT) activity was measured as previously described [16]. The rate of decrease in absorbance at 240 nm was measured as an index of H_2O_2 degradation by catalase. Superoxide dismutase (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline autooxidation in a spectrophotometer at 480 nm [17]. To determine glyoxalase I (GLO) activity we quantified the rate of formation of S-D-lactoylglutathione at 240 nm. The assay was carried out in 96-well microplates using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, Spectra Max 190). Briefly, 10 μL of 1 mM glutathione (GSH) and 2 mM methylglyoxal (MG), pre-incubated for 30 min at room temperature, in 50 mM sodium phosphate buffer (pH 7.0) were added to each well containing 190 μL samples (10 μg protein). The enzyme activity was calculated utilizing the molar extinction coefficient of 3300 M^{-1} cm^{-1} and expressed as units/mg protein, one unit being the amount of enzyme needed to produce 1 μmol/min of S-D-lactoylglutathione at 25 °C [18].

2.3.5. Enzyme linked immuno sorbent assay (ELISA) for CML

The wells of a microtiter plate were coated overnight with 0.1 μg protein in 0.1 mL of 50 mM sodium carbonate buffer (pH 9.6). Wells were washed three times with washing buffer (PBS containing 0.5% Tween 20), and then incubated with 0.5% gelatin for 3 h to block nonspecific binding. Thereafter, wells were washed again with washing buffer and incubated with 100 μL anti-CML (2G11) for 1 h. After being washed three times, wells were incubated with 100 μL of peroxidase-conjugated second antibody for 60 min. The reactivity of peroxidase was determined by incubation with *o*-phenylenediamine dihydrochloride (OPD) for 30 min. The reaction was stopped by addition of 50 μL sulphuric acid (3 M). Absorbance was read at 492 nm [19].

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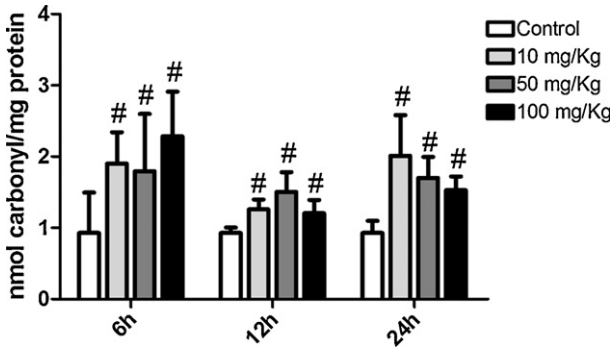

Fig. 1 – Circulating GA induces protein carbonylation. Glycolaldehyde was administered intravenously at the following concentrations: 0, 10, 50 or 100 mg/kg. Kidney was surgically removed after 6, 12 or 24 h. Data presented as mean ± SD ($n = 7$). #Different from respective control, $p < 0.05$.

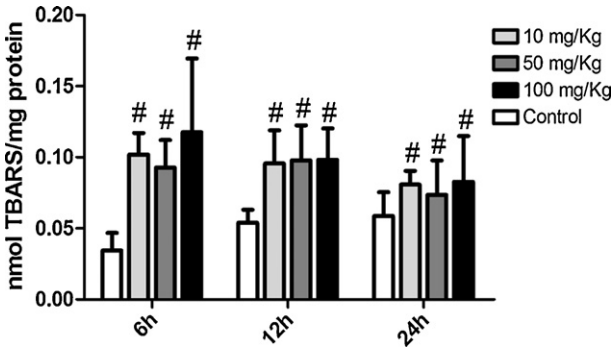

Fig. 3 – Lipoperoxidation increases after GA injection. The levels of thiobarbituric acid reactive species (TBARS) were assayed as an index of lipid peroxidation. At all doses, GA promoted lipid peroxidation, which was persistent up to 24 h after injection. Data presented as mean ± SD ($n = 7$). #Different from respective control, $p < 0.05$.

2.4. *Statistical analysis*

Results are expressed as mean ± SD. Data were analyzed by one-way ANOVA followed by Newman–Keuls' multiple comparisons test using software Prism 2.01 (GraphPad, San Diego, CA, USA). A p-value <0.05 was considered statistically significant.

3. Results

3.1. *Oxidative damage and redox status*

Animals that received intravenous injection of GA showed increased oxidative damage when compared to control rats. Fig. 1 shows the levels of protein carbonylation. All doses were capable of promoting protein carbonylation and this effect was sustained for 24 h.

The content of reduced thiol groups was decreased in the kidney of rats that received GA treatment. This oxidation of sulfhydryl was observed for all doses and all times analyzed (Fig. 2).

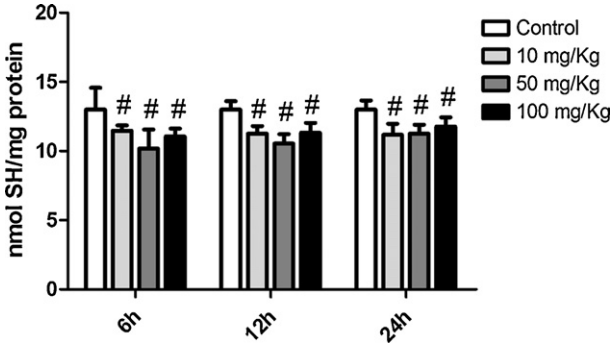

Fig. 2 – Glycolaldehyde lowers the content of reduced thiols in the kidney of Wistar rats. Animals received a single injection of GA. Samples were incubated with DTNB for 20 min. Absorbance was read at 412 nm. Data presented as mean ± SD ($n = 7$). #Different from respective control, $p < 0.05$.

Fig. 3 shows an increase in lipid peroxidation induced by GA. Although all doses promoted damage to lipids, after 24 h, levels of TBARS decreased to control levels suggesting clearance and/or a better response to the oxidative insult.

3.2. *Antioxidant enzymes*

Glycolaldehyde, at all tested concentrations, decreased SOD activity at 6 and 24 h after injection (Fig. 4a).

As shown in Fig. 4b, catalase activity was also decreased at 6 and 24 h after injection. Twelve hours after the injection of GA, kidney catalase activity was higher in rats that received 50 and 100 mg/kg, when compared to control.

The activity of glyoxalase I (GLO) was also assessed. This enzyme catalyzes the formation of S-D-lactoylglutathione from methylglyoxal and glutathione, leading to diminished concentrations of it substrate. We found decreased GLO activity by all doses of GA at 6 and 24 h after injection (Fig. 4c). However, no difference was found at 12 h.

3.3. *CML content*

Although protein carbonylation was evidenced, we did not observe any change in the protein content of N^{ε}-(carboxymethyl)lysine (Fig. 5).

4. Discussion

In the present study we evaluated the effects of circulating glycolaldehyde on redox status of the kidney. The physiological concentrations of GA have not been determined yet, although they are believed to range from 0.1 to 1 mM [10–12]. We injected GA at 10, 50 and 100 mg/kg. These doses were used because they would lead to blood concentrations ranging 1–20 mM, based on estimated blood volume [20].

Renal failure is a common complication in diabetic patients. Diabetic nephropathy results from a synergistic

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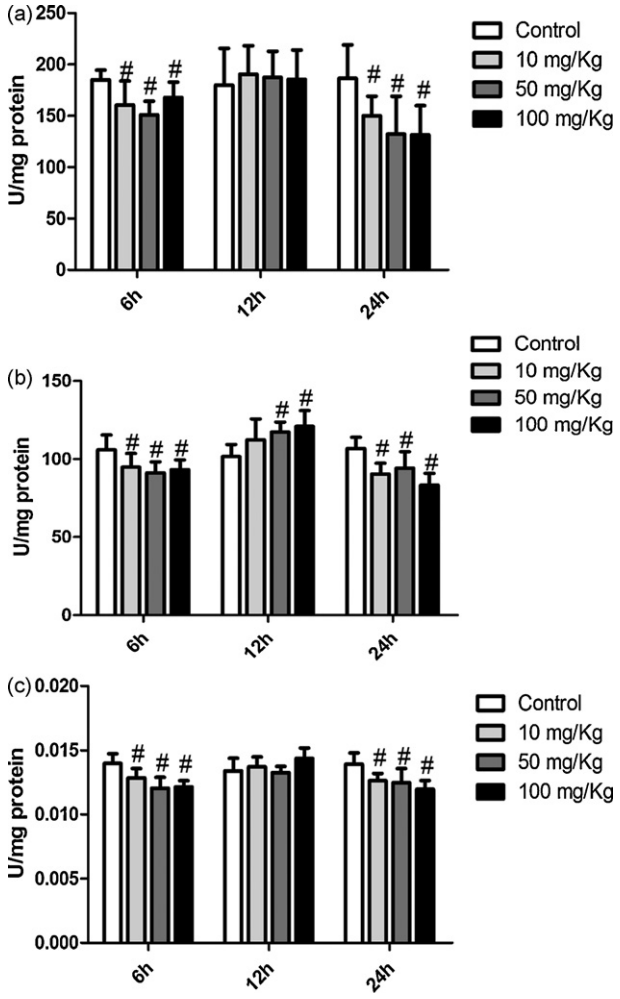

Fig. 4 – Glycolaldehyde decreases enzyme activities in the kidney. Wistar rats were killed 6, 12 or 24 h after GA injection. Superoxide dismutase (a), catalase (b) and glyoxalase I (c) were downregulated at 6 and 24 h after injection. Twelve hours after GA administration, CAT activity was increased in comparison to control group. Data presented as mean ± SD (n = 7). #Different from respective control, $p < 0.05$.

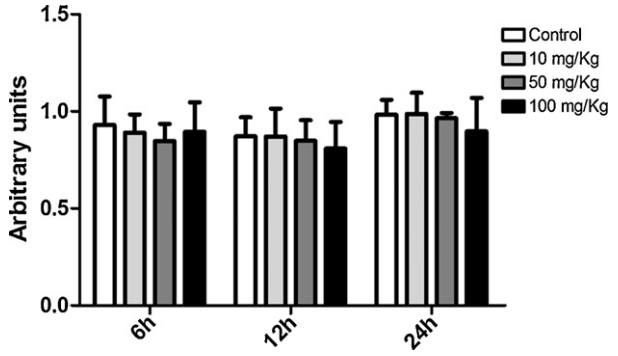

Fig. 5 – N^{ε}-(carboxymethyl)lysine content in renal proteins. Specific antibody (2G11) against CML was incubated with 0.1 μg protein. Peroxidase-conjugated second antibody was added and reactivity was determined by incubation with OPD. Data presented as mean ± SD (n = 7).

action of hemodynamic and metabolic factors [21]. Hemodynamic factors account for increased glomerular pressure [22] and expression of vascular endothelial growth factor [23], while metabolic factors account for increased reactive oxygen species [24] and AGEs [25]. Glycated proteins such as albumin [26] and fibrinogen [27] accumulate in the kidney. We previously demonstrated that fibrinogen is very susceptible to glycation by GA, which impairs its function and leads to formation of high molecular weight aggregates [8].

The formation of AGEs aggravates renal complications. Inhibition of this process ameliorates kidney function and diminishes albuminuria in diabetic animals [25]. Circulating AGEs are elevated in patients on peritoneal dialysis and with diabetes [28]. Interaction of AGEs with RAGE induces activation of NF-κB and elicits inflammation [5]. Such interaction also results in oxidative stress [29]. Diabetic mice with RAGE gene knock-out have a decrease in mitochondrial and cytosolic superoxide production [30].

Reducing sugars are capable of reacting with amino groups of proteins and thus impairing their function. Reaction of albumin with GA and other aldehydes diminishes its drug-binding capacity [7]. Low-density lipoproteins (LDL) are also susceptible to glycation by GA, in a reaction accelerated in the presence of Cu^{2+} [31]. There is evidence that glycated albumin [32] and glycated LDL [33] are involved in atherosclerotic events, enhancing macrophage inflammatory response.

A single injection of GA was capable of inducing carbonyl stress to proteins and of increasing lipoperoxidation (Figs. 1 and 3). Since GA can react with cysteine residues [34] it was of interest to evaluate the redox status of thiol groups in the kidney. We observed a 10–20% decrease in reduced thiol content (Fig. 2). Activities of SOD, CAT and GLO were decreased after GA injection (Fig. 4). The importance of the role played by GA in this setting still remains to be elucidated. It has been demonstrated that methylglyoxal decreases SOD activity *in vivo* and *in vitro* [35]. Superoxide dismutase catalyzes the dismutation of radical superoxide anion radical into oxygen and hydrogen peroxide. A decrease in CAT activity might be due to a direct inhibition of SOD by GA, as superoxide directly inhibits catalase [36]. Glyoxalase I plays an important role in detoxification of α-oxo-aldehydes, mainly glyoxal and methylglyoxal. Knockout of the gene responsible for GLO increases cell death after ischemia–reperfusion. When overexpressed, GLO ameliorates renal conditions after stress [37]. Decreased GLO activity can be implicated in diminished clearance of methylglyoxal which can accumulate and lead to formation of AGEs. Despite the increased oxidative stress observed in our results, attested by damage to proteins and lipids, as well as downregulation of antioxidant enzymes, we did not observe any changes any changes neither in CML (Fig. 5) levels nor in plasma levels of creatinine (data not shown). This is probably due to the short-term exposure of the animals to circulating GA.

In conclusion, our findings suggest that reducing sugars, such as glycolaldehyde, can promote intra-renal oxidative stress. Renal tissue damage resultant from augmented oxidative stress is evident prior to intra-renal accumulation

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of CML and might be an important factor in the genesis and development of diabetic nephropathy. Further studies are necessary for a better understanding of the molecular mechanisms involved in aldehydes-mediated oxidative damage.

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Conflict of interest

There are no conflicts of interest.

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3.2 Glycolaldehyde induces oxidative stress in the heart: a clue to diabetic cardiomyopathy?

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Glycolaldehyde Induces Oxidative Stress in the Heart: A Clue to Diabetic Cardiomyopathy?

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Abstract Cardiovascular complications account for 80% of the mortality related to diabetes mellitus. Hyperglycemia is believed to be the major culprit of angiopathy and cardiomyopathy. High glucose levels and oxidative stress cause elevation of Advanced Glycation End-products that are known to contribute to diabetic complications and correlate with many diseases. However, there are few reports describing the effects of glycating agents other than glucose. Here, we aimed to evaluate the effects of glycolaldehyde (GA) on oxidative stress parameters in the heart of Wistar rats. Male Wistar rats received a single injection of GA (10, 50 or 100 mg/Kg) and were sacrificed 6, 12 or 24 h after injection. As indexes of oxidative stress, we quantified protein carbonylation, lipid peroxidation and total reduced thiols. The activities of superoxide dismutase, catalase and glyoxalase I were assayed. Also, the content of N^{ε}-(carboxymethyl)lysine (CML) was quantified. Glycolaldehyde induced an imbalance in the redox status, with increased protein carbonylation and lipoperoxidation. Catalase and glyoxalase I had a decrease in their activities. Despite the oxidative stress, we observed no increase in CML content. These results suggest that short-chain aldehydes such as GA might have a significant role in the development of diabetic cardiomyopathy.

Keywords Glycolaldehyde · Cardiovascular disease · Glycation · Oxidative stress

Introduction

Cardiovascular disease (CVD) accounts for 80% of the mortality associated with diabetes mellitus (DM). Heart disease in DM affects function at different levels, promoting angiopathy and cardiomyopathy [1]. Type II diabetic patients with coronary artery disease, which causes the occlusion of the arteries that supply the heart, display more severe coronary atherosclerosis than non-diabetic subjects [2]. It is suggested that hyperglycemia is the main factor behind the majority of cardiovascular complication in DM, and blood glucose control reduces coronary atherosclerosis [3, 4]. Along with osmotic stress via the increased glucose flux through the polyol-sorbitol pathway [5], hyperglycemia also increases the formation of advanced glycation end-products (AGEs).

Advanced glycation end-products commonly arise from reaction of reducing sugars, such as glucose and short-chain aldehydes, with amino groups. Once formed, AGEs are very stable and often accumulate in the body. Diabetes [6], Alzheimer's disease [7] and the aging process [8] are closely associated with elevated levels of AGEs. Plasma levels of N^{ε}-(carboxymethyl)lysine (CML) are associated with liver failure [9] and chronic kidney disease [10].

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There is evidence that cardiac dysfunction in diabetes is associated with AGEs accumulation [11].

Many pathological effects of AGEs are due to interaction with receptor for AGEs (RAGE). In septic mice, CML interaction with RAGE, enhances inflammatory response and mortality by activating NFκB. Mice not expressing RAGE are protected from these effects [12]. Mitochondrial and cytosolic superoxide formation is attenuated after RAGE deletion in diabetic mice [13]. Superoxide radical is believed to participate in the coronary vasoconstriction induced by CML [14]. Furthermore, oxidative stress is a common component of the diabetic heart, evidenced by increased lipoperoxidation and protein carbonylation [15–17].

Glycolaldehyde (GA) is a short-chain aldehyde formed as a by-product of protein glycation [18] and myeloperoxidase (MPO) activity upon amino acids [19]. GA reacts with amino groups forming a Schiff base that rearranges to form stable Amadori products that lead to AGEs. Glycolaldehyde itself induces oxidative stress and apoptosis [20] and impair functions of fibrinogen [21] and albumin. The most prevalent AGEs derived from GA are GA-pyridine and CML [22]. Although there is evidence demonstrating a pathophysiological role for GA, its physiological concentrations have not been determined yet. It has been estimated that GA concentration ranges from 0.1 to 1 mM [23–25].

Despite several reports describing the effects of glycated molecules on redox status and cell homeostasis, there is little investigation on the direct effects of glycating agents. Thus, in this work, we aim to investigate the acute effects of GA on oxidative stress parameters in the heart of Wistar rats, in order to evaluate the possible effects of short-term glycoxidation events.

Materials and Methods

Animals and Chemicals

Three-month-old adult male Wistar rats (280–320 g) were obtained from our own breeding colony. They were caged in groups of five with free access to water and food and were maintained on a 12 h light–dark cycle (lights on at 7 a.m.), at a temperature-controlled colony room (23 ± 1°C). These conditions were maintained constant throughout the experiments. All experimental procedures were performed in accordance with the National Institute of Health Guides for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior recommendations for animal care.

All chemicals were purchased from Sigma (St. Louis, USA).

Treatments

Animals were anesthetized (ketamin 100 mg/Kg and xylazin 10 mg/Kg) and treated with a single injection of GA via the dorsal vein of the penis, in different doses (10, 50 and 100 mg/Kg) in a volume range of 120–150 µL. Control group received 130 µL of NaCl 0,9%. The doses were calculated in order to have GA concentrations in blood ranging from 1 to 20 mM. Calculations were based on average blood volume of Wistar rats [26].

Oxidative Stress and Antioxidant Enzyme Analysis

Animals were sacrificed at 6, 12 or 24 h after injection. Blood was collected and plasma separated. The heart was dissected out in ice and immediately stored at −80°C for posterior analysis. Homogenates were centrifuged (1,000*g*, 10 min at 4°C) to remove cellular debris. Supernatants were used to all biochemical assays described herein. For ELISA, supernatants were centrifuged once more (10,000*g*, 10 min at 4°C) and were diluted in phosphate saline buffer containing 0.05% sodium azide, 0.5% Triton X-100 and a protease inhibitor cocktail (pH 7.4).

Measurement of Protein Carbonylation

The oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with 2,4-dinitrophenylhydrazine (DNPH). Proteins were precipitated by the addition of 20% trichloroacetic acid (TCA) and resuspended in 10 mM DNPH, and the absorbance read at 370 nm [27]. Results were expressed as nmol carbonyl/mg protein.

Measurement of Thiobarbituric Acid Reactive Species (TBARS)

As an index of lipoperoxidation, we detected thiobarbituric acid reactive species (TBARS) formation through a hot and acidic reaction. This is widely adopted as a method for measurement of lipid redox state, as previously described [28]. Briefly, the samples were mixed with 0.6 mL of 10% TCA and 0.5 mL of 0.67% thiobarbituric acid and then heated in a boiling water bath for 25 min. TBARS were determined by absorbance in a spectrophotometer at 532 nm. We have obtained TBARS concentration in the samples from a calibration curve that was performed using 1,1,3,3-tetramethoxypropane as standard, which was subjected to the same treatment as that applied to the supernatants of the samples. Results are expressed as nmol TBARS/mg protein.

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Measurement of Total Reduced Thiol Content

To quantify the content of reduced thiol, samples were diluted in 10 mM phosphate buffer (pH 7.4), followed by the addition of 0.01 M 5,5′-dithionitrobis 2-nitrobenzoic acid (DTNB) in ethanol. The intense yellow color was developed and read at 412 nm after 20 min. A blank sample was run simultaneously, except for the absence of DTNB. Protein thiol content was calculated after subtraction of the blank absorbance utilizing the molar extinction coefficient of 13,600 M^{-1} cm^{-1} [29].

Measurement of Enzyme Activities

Catalase (CAT) activity was measured as previously described [30]. The rate of decrease in absorbance at 240 nm was measured as an index of H_2O_2 degradation by catalase. One unit of CAT was considered to be the amount of enzyme needed to degrade 1 μmol/min H_2O_2 at 25°C. Superoxide dismutase (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm [31]. To determine glyoxalase I (GLO) activity, we quantified the rate of formation of S-D-Lactoylglutathione at 240 nm. The assay was carried out in 96-well microplates using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, Spectra Max 190). Briefly, 10 μL of 1 mM glutathione (GSH) and 2 mM methylglyoxal (MG), pre-incubated for 30 min at room temperature, in 50 mM sodium phosphate buffer (pH 7.0) were added to each well containing 190 μL samples (10 μg protein). The enzyme activity was calculated utilizing the molar extinction coefficient of 3,300 M^{-1} cm^{-1} and expressed as units/mg protein, one unit being the amount of enzyme needed to produce 1 μmol/min of S-D-Lactoylglutathione at 25°C [32].

Enzyme-Linked Immuno Sorbent Assay (ELISA) for CML

The wells of a microtiter plate were coated overnight with 0.1 μg protein in 0.1 mL 50 mM sodium carbonate buffer (pH 9.6). Wells were washed three times with washing buffer (PBS containing 0.5% Tween 20) and then incubated with 0,5% gelatin for 3 h to block non-specific binding. After, wells were washed again with washing buffer and incubated with 100 μL anti-CML (2G11) for 1 h. After being washed three times, wells were incubated with 100 μL of peroxidase-conjugated second antibody for 60 min. The reactivity of peroxidase was determined by incubation with *o*-phenylenediamine dihydrochloride (OPD) for 30 min. The reaction was stopped with addition of 50 μL 3 M sulphuric acid. Absorbance was read at 492 nm [33].

Statistical Analysis

Results are expressed as mean ± SD. Data were analyzed by one-way ANOVA followed by Newman–Keuls' multiple comparisons test using software Prism 2.01 (GraphPad, San Diego, CA, USA). A P-value < 0.05 was considered statistically significant.

Results

Redox Status

All doses of GA induced protein carbonylation. These modifications were persistent up to 24 h (Fig. 1). Damage to lipids was also assessed, and GA promoted an increase in the levels of TBARS persistent up to12 h after injection. Figure 2 shows the levels of lipoperoxidation in the heart of treated rats. Along with protein damage, reduced thiol content was also quantified. After 12 and 24 h, the content of reduced thiol was decreased in the heart of rats treated with GA (Fig. 3).

Enzymes Activities

All enzymes assayed were modulated by the treatment with GA. Superoxide dismutase showed an increase in activity at 6 h and a decrease at 24 h after GA injection (Fig. 4a). The injection of GA induced a decrease in catalase activity that was persistent up 12 h after treatment (Fig. 4b).

Glyoxalase I had an increase in its activity in rats treated with GA, but only 6 h after injection (Fig. 4c).

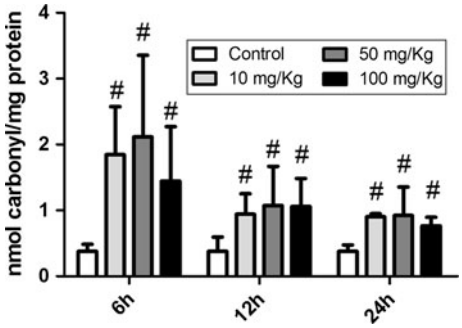

Fig. 1 Circulating GA induces protein carbonylation in the heart. Glycolaldehyde was administered intravenously at the following concentrations: 0, 10, 50 or 100 mg/Kg. Heart was surgically removed after 6, 12 or 24 h. Data presented as mean ± SD ($n = 7$). # Different from respective control, $P < 0.05$

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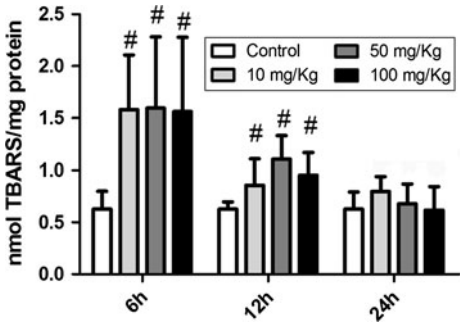

Fig. 2 Lipid peroxidation increases after GA injection. The levels of thiobarbituric acid reactive species (TBARS) were assayed as an index of lipid peroxidation. All doses of GA increased the levels of TBARS up to 12 h after injection. Data presented as mean ± SD ($n = 7$). # Different from respective control, $P < 0.05$

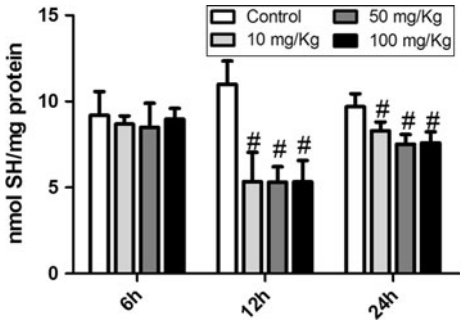

Fig. 3 Glycolaldehyde promotes oxidation of thiols in the heart of Wistar rats. Animals received a single injection of GA. Samples were incubated with DTNB for 20 min. Absorbance was read at 412 nm. All doses of GA induced oxidation of thiols observed 12 h after injection. Data presented as mean ± SD ($n = 7$). # Different from respective control, $P < 0.05$

CML Content

Although GA can form N^{ε}-(carboxymethyl)lysine, we did not observe any increase in the content of CML (Fig. 5).

Discussion

In this work, we aimed to investigate the effects of circulating glycolaldehyde on oxidative stress parameters of the heart. Here, we show for the first time the acute effects of circulating GA on redox status of the heart.

Glycolaldehyde can arise from several sources and the pivotal ones include glucose auto-oxidation and MPO activity [18, 19]. The importance of this compound is reinforced by data, which show the increased risk of cardiovascular complications in diabetic patients and that increased MPO activity in heart failure patients predicts mortality [34]. Moreover, GA can act as a glycation agent and thus, promoting the generation of AGEs, which can bind to membrane receptors and trigger cellular signaling

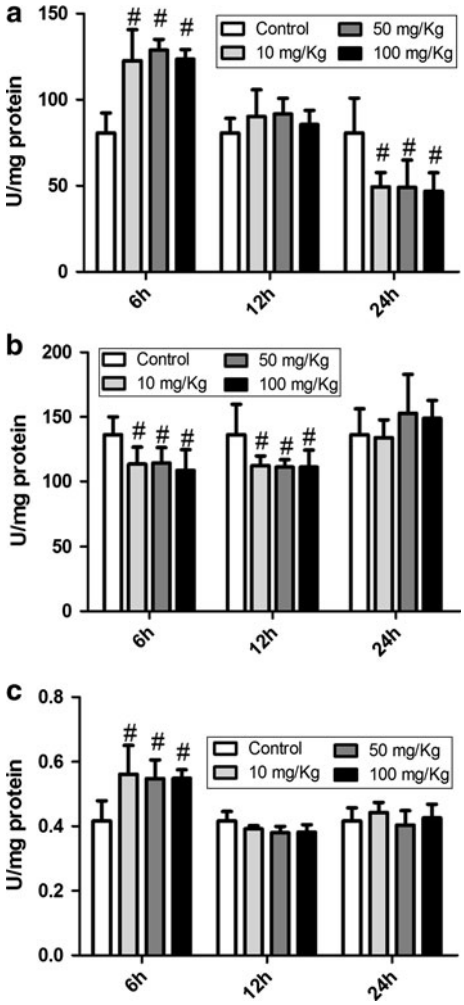

Fig. 4 Glycolaldehyde decreases enzyme activities in the heart. Wistar rats were killed 6, 12 or 24 h after GA injection. Superoxide dismutase (**a**) and glyoxalase I (**c**) had their activities increased 6 h after injection. Activity of catalase was decreased 6 and 12 h after injection (**b**). Data presented as mean ± SD ($n = 7$). # Different from respective control, $P < 0.05$

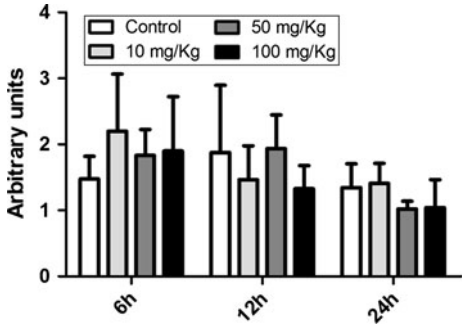

Fig. 5 N^{ε}-(carboxymethyl)lysine content in the heart of Wistar rats injected with glycolaldehyde. Specific antibody (2G11) against CML was incubated with 0.1 µg protein. Peroxidase-conjugated second antibody was added and reactivity was determined by incubation with OPD. Data presented as mean ± SD ($n = 7$)

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[35]. In fact, it was already demonstrated in a model of atherosclerosis in mice that treatment with soluble RAGE can avoid the development of plaques in the aortic sinus [36].Despite these evidences, there is no work describing the effects of circulating GA in heart oxidative parameters.

We observed an increase in oxidation of proteins and lipids (Figs. 1, 2). Moreover, GA modulated the activities of SOD, CAT and GLO (Fig. 4). The higher SOD activity, 6 h after GA injection, could increase hydrogen peroxide levels. Because CAT activity was decreased until 12 h after injection, the defense against hydrogen peroxide could be impaired, leading to higher concentrations of this reactive molecule. The increase in SOD activity could also be a response to higher levels of radical superoxide. Also, it has been demonstrated that radical superoxide directly inhibits catalase [37]. Thus, reaction of GA with proteins and the action of oxygen reactive species (ROS) could promote the carbonylation observed.

A decrease in reduced thiol content was also observed 12 and 24 h after injection. GA reacts mainly with lysine and arginine, but also with cysteine residues [38]. Cysteine residues, in proteins or glutathione (GSH), act as ROS scavengers, regulating cellular redox status [39]. Nevertheless, cysteine residues could also be acting as transient nucleophiles [40], a fact that explains why the oxidation was observed only 12 h after injection. The lower levels of thiols might be a reflection of the increase in GLO activity. GLO plays a major role in the clearance of α-oxoaldehydes like methylglyoxal (MG) and glyoxal. Furthermore, oxidative stress events reduce GSH levels, impairing clearance of MG [41]. Animal models of DM indicate that oxidative damage is frequent component of the disease. Increased carbonyl content of the heart has been observed in diabetic animals [15, 17, 42]. Furthermore, antioxidant treatments restore cardiac function after diabetes induction [43, 44]. We also assessed the plasma activities of glutamic oxaloacetic transaminase and glutamic pyruvic transaminase (data not shown), which are common markers of hepatic and heart dysfunction. However, we did not observe any alterations.

Despite the oxidative damage and enzyme modulation, no increase in CML content was found. This could be due to the short-term exposure or to an efficient clearance of GA and AGEs. Another possible explanation is the generation of GA-pyridine, a type of GA-modification that cannot be detected by antibody 2G11 [45]. This modification is implied in atherosclerosis and was detected in human atherosclerosis lesions [46].

The acute model presented in this work has its flaws when it fails to show organ dysfunction. However, in a chronic treatment, one should take in account the greater participation of AGEs and other molecules that would be increased due to the long-term exposure to GA. The present work shows for the first time the acute effects of GA on oxidative stress in the heart, which might provide a better understanding of the development of diabetes complications.

In conclusion, even short-term exposures to GA increased markers of oxidative stress, suggesting that cumulative events of hyperglycemia and inflammation, which favor GA formation, might damage cardiac tissue and thus lead to dysfunction.

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3.3 Oxidative damage in the liver of rats treated with glycolaldehyde

Artigo submetido ao periódico *Journal of Biochemical and Molecular Toxicology*.

Oxidative damage in the liver of rats treated with glycolaldehyde

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Key-words: glycolaldehyde; liver disease; glycation; oxidative stress

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Abstract

Liver diseases are often associated with hyperglycemia, inflammation and oxidative stress. These conditions, commonly associated with diabetes mellitus and obesity, facilitate the formation of Advanced Glycation End-products (AGEs). These products are known to impair protein function and promote inflammation. Accumulation of AGEs such as *N*ε-(carboxymethyl)lysine (CML) is related to chronic liver diseases and their severity. Although several reports suggest a crucial role of AGEs in liver failure, there is little investigation on the direct effects of reducing sugars, precursors of AGEs, on the onset and progression of liver failure. In this work we investigate the effects of intravenously administrated glycolaldehyde (GA), a short-chain aldehyde, on oxidative parameters in the liver of Wistar rats. Animals received a single injection of GA (10, 50 or 100 mg/Kg) and were sacrificed at 6, 12 or 24 hours after. Levels of protein carbonyl, lipid peroxidation and reduced thiol were quantified. The activities of catalase, superoxide dismutase and glyoxalase I were also assessed. The amount of CML was quantified with specific antibody. There was an increased in oxidative stress markers in the liver of GA-treated rats. Glycolaldehyde induced a decrease in the activities of all enzymes assayed. Also, all tested doses led to an increase in CML content. Our data suggest that GA might play an important role in liver diseases, through impairment of antioxidant defenses and generation of AGEs.

INTRODUCTION

The liver plays a main role on metabolism. Regulation of glucose levels and synthesis of lipoproteins and fatty acids are among its most important functions. Impairment of liver function is known to be crucial in diabetes, cirrhosis and steatohepatitis. These diseases are related to insulin resistance, obesity and abusive alcohol ingestion (1-2). In type 1 diabetic rats, alanine transaminase (ALT) and alkaline phosphatase (ALP) are elevated in plasma and these animals show an increase of apoptotic cells in the liver (3). In non-alcoholic hepatosteatosis (NASH), insulin resistance, inflammation and oxidative stress are believed to be major culprits in disease development (4). Several models of liver disease suggest a crucial participation of oxidative stress in liver failure (5-6). This role is corroborated in many reports by the beneficial effects of antioxidant therapy. Treatment with N-acetylcysteine reduces fibronectin deposition and attenuates oxidative damage in rats with dimethylnitrosamine-induced liver fibrosis (7). Some plant extracts act as hepatoprotectors in different animal models, mainly by reducing oxidative damage (8-11).

Another common feature of liver diseases is the increase in Advanced Glycation End-products (AGEs). AGEs are formed through reaction of reducing sugars and amino groups, forming a Schiff base that rearranges to more stable Amadori products and later lead to AGEs (12). Hepatic endothelial and Kupfer cells are responsible for AGEs clearance via endocytosis mediated by the scavenger receptor (13). Liver transplantation in cirrhotic patients lowers the levels of plasma N^{ε}-(carboxymethyl)lysine (CML) (14). Plasma CML levels are also correlated with the severity of cirrhosis (15). Serum glyceraldehyde-derived AGE is elevated in patients

with NASH in comparison to healthy subjects and those with simple steatosis (16). Activation of the receptor for AGEs (RAGE) promotes migration of activated hepatic stellate cells (HSCs), which are the main extracellular matrix-producing cells in the liver. AGEs upregulate fibrogenic genes in HSCs in an oxidative process that can be prevented by antioxidants (17). Deletion of RAGE in diabetic mice prevents mitochondrial and cytosolic excess generation of superoxide (18). Recent report demonstrates that AGEs upregulate RAGE in quiescent and activated HSCs, leading to ROS production via activation of NADPH oxidase (19). CML interacts with RAGE and activates NF-κB, increasing inflammatory response in septic mice (20). RAGE -/- mice are protected from such activation and present improvement in survival.

Glycolaldehyde is a by-product of non-enzymatic glycosylation (21) and of the myeloperoxidase in neutrophils (22). It rapidly reacts with amino groups, mainly lysine and arginine, leading to formation of AGEs such as CML and GA-pyridine (23). In MCF7 human breast cancer cells, GA decreases cell viability, induces superoxide radical production and increases lipid peroxidation (24). We previously demonstrated that GA promotes protein carbonylation and impairs fibrinogen coagulation (25). Although there is evidence demonstrating a pathophysiological role for GA, its physiological concentrations have not been determined yet. It has been estimated that GA concentration ranges from 0.1 to 1 mM (26-28).

Despite a huge interest in elucidating the mechanisms that directly link AGEs and many diseases, and several evidences of the involvement of oxidative stress in these pathologies, little is known about AGEs precursors, such as GA, and their effect on oxidative stress and biochemical parameters. Thus, in this study we aimed to

evaluate the acute effects of a single intravenous injection of GA on oxidative parameters in the liver of Wistar rats.

MATERIALS AND METHODS

Animals and Chemicals

Adult male Wistar rats (280 – 320g) were obtained from our own breeding colony. They were caged in groups of five with free access to water and food and were maintained on a 12h light-dark cycle (lights on at 7 a.m.), at a temperature controlled colony room (23 ± 1°C). These conditions were maintained constant throughout the experiments. All experimental procedures were performed in accordance with the National Institute of Health Guides for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior recommendations for animal care.

All chemicals were purchased from Sigma (St. Louis, USA).

Treatments

Animals were anesthetized (ketamin 100 mg/Kg and xylazin 10 mg/Kg) and treated with a single injection of GA via the dorsal vein of the penis, in different doses (10, 50 and 100 mg/Kg) in a volume range of 120 – 150 µL. Control group received 130 µL of NaCl 0.9%.

Oxidative stress and antioxidant enzymes analysis

Animals were sacrificed at 6, 12 or 24h after injection. Blood was collected and plasma separated. The liver was dissected out in ice and immediately stored at -80ºC

for posterior analysis. Homogenates were centrifuged (1000*g*, 10min at 4ºC) to remove cellular debris. Supernatants were used to all biochemical assays described herein. Samples for quantification of CML content were diluted in phosphate saline buffer containing 0.05% sodium azide, 0.5% Triton X-100 and a protease inhibitor cocktail (pH 7.4).

Measurement of protein carbonyl

The oxidative protein damage was measured by the quantification of carbonyl groups based on the reaction with 2,4-dinitrophenylhydrazine (DNPH). Proteins were precipitated by the addition of 20% trichloroacetic acid (TCA) and resuspended in 10 mM DNPH and the absorbance read at 370 nm (29). Results were expressed as nmol carbonyl/mg protein.

Measurement of thiobarbituric acid reactive species (TBARS)

As an index of lipid peroxidation we detected thiobarbituric acid reactive species (TBARS) formation through a hot and acidic reaction. This is widely adopted as a method for measurement of lipid redox state, as previously described (30). Briefly, the samples were mixed with 0.6 mL of 10% TCA and 0.5 mL of 0.67% thiobarbituric acid and then heated in a boiling water bath for 25 min. TBARS were determined by absorbance in a spectrophotometer at 532 nm. We have obtained TBARS concentration in the samples from a calibration curve that was performed using 1,1,3,3-tetramethoxypropane as standard, which was subjected to the same

treatment as that applied to the supernatants of the samples. Results are expressed as nmol TBARS/mg protein.

Measurement of total reduced thiol content

To quantify the content of reduced thiol, samples were diluted in 10mM phosphate buffer (pH 7.4), followed by the addition of 0.01 M 5,5′-dithionitrobis 2-nitrobenzoic acid (DTNB) in ethanol. The intense yellow color was developed and read at 412 nm after 20 min. A blank sample was run simultaneously, except for the absence of DTNB. Protein thiol content was calculated after subtraction of the blank absorbance, utilizing the molar extinction coefficient of 13,600 M^{-1} cm^{-1} (31).

Assay of Enzymes Activities

Catalase (CAT) activity was measured as previously described (32). The rate of decrease in absorbance at 240 nm was used as an index of H_2O_2 degradation by catalase. One unit of CAT was considered to be the amount of enzyme needed to degrade 1 µmol/min H_2O_2 at 25ºC. Superoxide dismutase (SOD) activity was assessed by quantifying the inhibition of superoxide-dependent epinephrine auto-oxidation in a spectrophotometer at 480 nm (33). To determine glyoxalase I (GLO) activity we quantified the rate of formation of S-D-Lactoylglutathione at 240 nm. The assay was carried out in 96-well microplates using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, Spectra Max 190). Briefly, 10 µL of 1 mM glutathione (GSH) and 2 mM methylglyoxal (MG), pre-incubated for 30 min at room temperature, in 50 mM sodium phosphate buffer (pH 7.0) were

added to each well containing 190 µL samples (10 µg protein). The enzyme activity was calculated utilizing the molar extinction coefficient of 3300 M^{-1} cm^{-1} and expressed as units/mg protein, one unit being the amount of enzyme needed to produce 1 µmol/min of S-D-Lactoylglutathione at 25°C (34).

Enzyme Linked Immuno Sorbent Assay (ELISA) for CML

The wells of a microtiter plate were coated overnight with 0.1 µg protein in 0.1 mL 50mM sodium carbonate buffer (pH 9.6). Wells were washed three times with washing buffer (PBS containing 0.5% Tween 20), and then incubated with 0.5% gelatin for 3 hours to block nonspecific binding. After, wells were washed again with washing buffer and incubated with 100 µL anti-CML (2G11) for 1 hour. After being washed three times, wells were incubated with 100 µL of peroxidase-conjugated second antibody for 60 minutes. The reactivity of peroxidase was determined by incubation with *o*-phenylenediamine dihydrochloride (OPD) for 30 minutes. The reaction was stopped with addition of 50 µL 3M sulphuric acid. Absorbance was read at 492 nm (35).

Statistical Analysis

Results were expressed as mean ± SEM. Data were analyzed by one-way ANOVA followed by Newman-Keuls' multiple comparisons test using software Prism 2.01 (GraphPad, San Diego, CA, USA). A p-value <0.05 was considered statistically significant.

RESULTS

Redox Status

All doses of GA induced protein carbonylation. These modifications were persistent up to 24h (Fig. 1). Damage to lipids was also assessed and GA promoted an increase in the levels of TBARS. Figure 2 shows the levels of lipid peroxidation in the liver of treated rats. Along with protein damage, reduced thiol content was also quantified. As presented in figure 3, animals treated with glycolaldehyde showed lower levels of reduced thiols. This was observed only 12 and 24 hours after injection.

Assay of Enzymes Activities

All enzymes assayed had a decreased in their specific activity after the treatment with GA. Superoxide dismutase, which catalyzes the dismutation of radical superoxide anion radical into oxygen and hydrogen peroxide had a decrease in its activity 6 and 12 hours after GA injection (Fig. 4a). Catalase, responsible for dealing with hydrogen peroxide, was also decreased at the same times (Fig. 4b), although the lower dose had no effect at 6h. The activity of GLO was lower in comparison to control group only 12 hours after GA injection (Fig. 4c). Although all concentrations of GA could affect enzyme function, activities were restored 24 hours after injection.

CML Content

The intravenous administration of GA promoted the formation of the Advanced Glycation End-Product CML (Fig. 5). All tested concentrations of GA induce the formation of CML. Twelve and 24h after injection, the amount of CML was quantified in the liver was dose-dependent.

DISCUSSION

Liver disease is often associated with fatty acid accumulation and cirrhosis. Such conditions are closely related to diabetes mellitus and metabolic syndrome. These conditions favor the formation of AGEs and the establishment of oxidative stress. In this work we show that intravenously administrated glycolaldehyde induces oxidative damage to proteins and lipids, and also decreases antioxidant enzymes activities. The doses of GA were chosen in order to obtain concentrations of circulating GA ranging from 1 to 20 mM, according to estimated blood volume of the animals (36).

Glycolaldehyde is a short-chain aldehydes that reacts mainly with lysine and arginine residues (23). Glycolaldehyde also reacts with cysteine residues (37), which might explain the lower levels of reduced thiols in the rats treated with GA (Fig. 3). Moreover, GA modulated the activities of SOD, CAT and GLO. The lower activity of SOD could raise anion superoxide levels and lead to direct inhibition of CAT (38). Methylglyoxal (MG), which reacts with arginine and lysine just as GA, inhibits liver SOD *in vivo* and *in vitro* (39). This could explain the inhibition of SOD induced by GA administration. Furthermore, the observed inhibition of GLO might lead to increased levels of MG that can inhibit SOD. Glyoxalase I converts the hemithioacetal adduct between glutathione and methylglyoxal into S-D-Lactoylglutathione. Oxidative stress events impair glutathione levels, reducing the clearance of MG (40). Despite the oxidative damage, no alteration in liver function was observed, as assessed by glutamic oxaloacetic transaminase and glutamic pyruvic transaminase (data not shown). This could be due to the short-term exposure to GA. However, even a short-term exposure was capable of increasing the CML

content (Fig. 5). Plasma CML levels correlate with liver cirrhosis and its severity (15). Liver transplantation in cirrhosis patients decreases plasma CML levels (14). The *N*ε-(carboxymethyl)lysine levels are also elevated in diabetic and peritoneal dialysis patients (41). Diabetes also increases CML content in soleus muscle (42) and vasculature (43). Moreover, CML levels are associated with chronic kidney disease (44).

The observed raise in CML levels can partly explain the high levels of protein carbonylation, as CML has a carbonyl group in its structure. Nonetheless, it remains unclear the extent of protein carbonylation that is due to formation of CML and to other oxidative processes. It seems, although, that the lower activities of antioxidant enzymes might have favored the observed redox imbalance.

In summary, our results show that circulating GA induces an oxidative state in the liver, which might contribute to the development of chronic liver diseases such as steatosis and cirrhosis. Cumulative events of glycoxidation, which raise the levels of GA and other aldehydes, might also contribute to the onset of common liver dysfunction observed in these complications, as well as in diabetes. For the first time it is shown that GA can promote accumulation of CML in the liver. With circulating levels ranging from 1 to 20 mM, GA induced oxidative damage to proteins and lipids and modulated the activities of SOD, CAT and GLO. The cumulative effects of such events, combined with genetic predisposition and other environmental conditions might lead to the progression of liver dysfunction and culminate in liver disease. Further work is necessary to elucidate the molecular mechanisms of short-chain aldehydes in liver pathologies.

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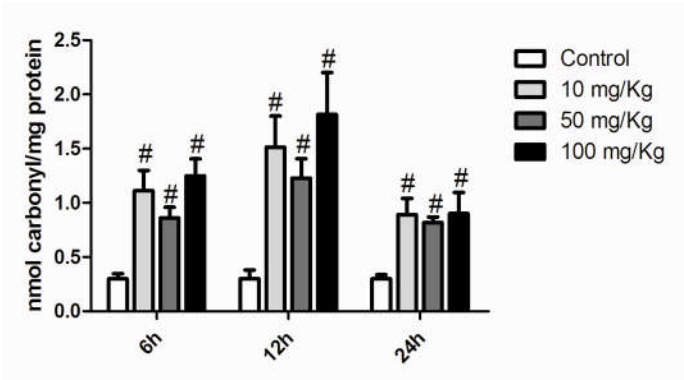

Fig. 1. Circulating GA induces protein carbonylation. Glycolaldehyde was administered intravenously at the following concentrations: 0, 10, 50 or 100 mg/Kg. Liver was surgically removed after 6, 12 or 24h. Data presented as Mean ± SEM (n=7). # different from respective control, $p<0.05$.

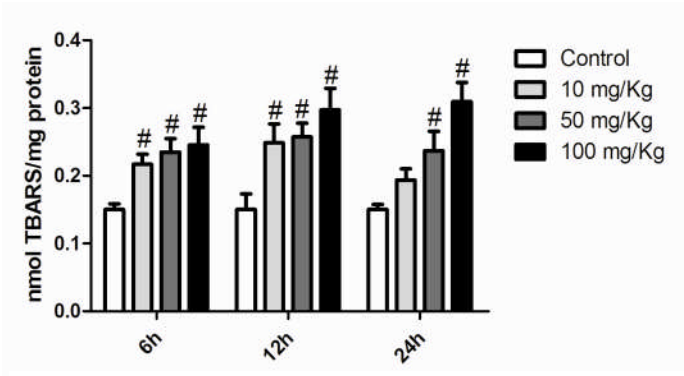

Fig. 2. Lipid peroxidation increases after GA injection. The levels of thiobarbituric acid reactive species (TBARS) were assayed as an index of lipid peroxidation. At all doses, GA promoted lipid peroxidation, which was persistent up to 24h after injection. Data presented as Mean ± SEM (n=7). # different from respective control, $p<0.05$.

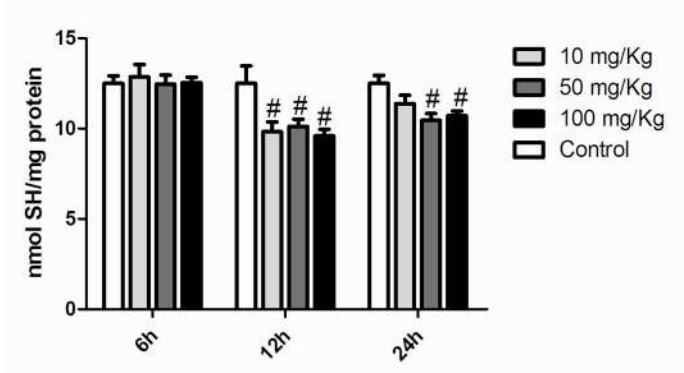

Fig. 3. Glycolaldehyde promotes oxidation of thiols in the liver of Wistar rats. Animals received a single injection of GA. Samples were incubated with DTNB for 20 minutes. Absorbance was read at 412 nm. Data presented as Mean ± SEM (n=7). # different from respective control, $p<0.05$.

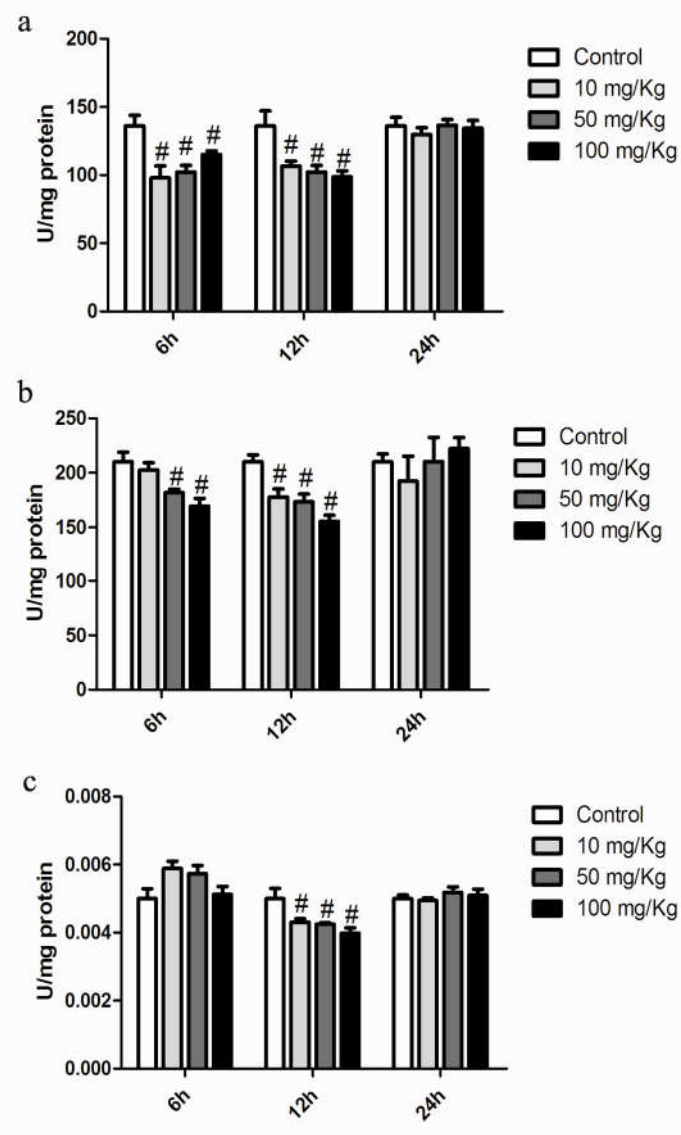

Fig. 4. Glycolaldehyde decreases enzyme activities in the liver. Wistar rats were killed 6, 12 or 24h after GA injection. Superoxide dismutase (a) and catalase (b) had a decrease in activity 6 and 12h after injection. Glyoxalase I was only altered 12h after GA injection. Data presented as Mean ± SEM (n=7). # different from respective control, $p<0.05$.

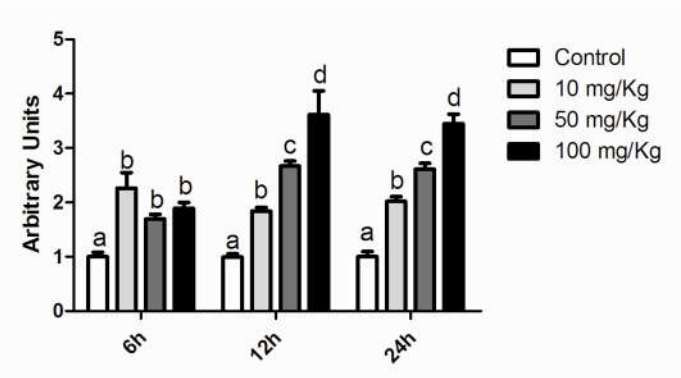

Fig. 5. *N*ε-(carboxymethyl)lysine content in the liver. Specific antibody (2G11) against CML was incubated with 0,1µg protein. Peroxidase-conjugated second antibody was added and reactivity was determined by incubation with OPD. Data presented as Mean ± SEM (n=7). Different letters indicate significant difference between groups. Each time was analyzed independently, $p<0.05$.

4. DISCUSSÃO

No presente trabalho, mostramos que uma única injeção de glicolaldeído, nas doses de 10, 50 e 100 mg/Kg, altera parâmetros de estresse oxidativo em ratos machos adultos. Como parâmetros de dano oxidativo, observamos aumento nos níveis de carbonilação proteica e peroxidação lipídica no rim, fígado e coração dos animais. Esse aumento nos marcadores de dano oxidativo foi acompanhado por uma redução nos níveis de tióis reduzidos, além da modulação das enzimas SOD, CAT e GLO.

Aldeídos como o GA são derivados do metabolismo da glicose, além de rearranjos de produtos da reação não enzimática da glicose com proteínas. A taxa de formação é dependente da concentração de glicose (Nagai *et al.*, 2005), portanto encontra-se aumentada durante o DM. Apesar de sua importância, os níveis fisiológicos de GA ainda não foram determinados. Estima-se que as concentrações fisiológicas variem de 0,1 a 1mM (Ukeda *et al.*, 1997; Morgan, Dean & Davies, 2002; Brown, Dean & Davies, 2005). Neste trabalho utilizamos doses que, conforme volemia aproximada dos animais (Lee & Blaufox, 1985), promoveriam doses de GA circulante variando de 1 a 20 mM.

O GA, assim como MG e glioxal, tem afinidade com resíduos de lisina e arginina. A ligação destes aldeídos em resíduos específicos pode levar a mudanças na atividade de proteínas. Quando a albumina é incubada com estes aldeídos, além da formação de AGEs, ocorre uma redução na capacidade desta proteína se ligar às drogas varfarina e cetoprofeno (Mera *et al.*, 2010). Em um trabalho do nosso grupo foi demonstrado que o GA reage com fibrinogênio, alterando tanto o tempo de formação do coágulo quanto sua resistência à digestão (Andrades *et al.*, 2009).

Quando glicada por ribose ou MG, a apoproteína A-I apresenta redução na promoção do transporte de colesterol de células como macrófagos e monócitos (Hoang *et al.*, 2007). Assim, é plausível que a redução na atividade das enzimas SOD, CAT e GLO observada no rim e no fígado seja devida a uma reação direta com o GA.

Quanto ao coração a atividade da CAT estava diminuída seis e doze horas após a injeção de GA, enquanto SOD e GLO apresentaram um aumento em suas atividades no tempo de 6 horas. Este comportamento sugere uma diferença na suscetibilidade à ação do GA. Entretanto, não podemos precisar se essa suscetibilidade é quanto à ação direta do GA ou quanto a ação dos AGEs. No que se trata da ação direta do aldeído, ela pode ser atenuada por moléculas que atuam "sequestrando" o GA e impedindo sua reação com outras biomoléculas. A GSH pode se ligar a aldeídos pelo seu resíduo de cisteína e a piridoxamina (vitamina B6) também tem esta propriedade (Onorato *et al.*, 2000). No caso da piridoxamina, sua atividade é devida a seu grupo amino (NH_2). Ao agir através da formação de AGEs, o principal mecanismo descrito é a interação com RAGE. A ligação AGE/RAGE aumenta a expressão da molécula de adesão de célula vascular-1 em cultura de endotélio e em camundongos (Schmidt *et al.*, 1995). Da mesma forma, interação com RAGE eleva a produção de radical superóxido em monócitos (Ding *et al.*, 2007) e nas mitocôndrias de células renais (Coughlan *et al.*, 2009). Deste modo, através do RAGE, os AGEs formados a partir do GA podem alterar a expressão de determinadas proteínas, além de promover a geração de ERO.

Em qualquer das situações e considerando a simultaneidade de ambas, a redução na atividade das enzimas antioxidantes acarreta em uma ineficiência na eliminação de ERO. O ânion superóxido é formado sob condições normais e é

bastante reativo. Quando a atividade da SOD é insuficiente para lidar com o superóxido formado, o excesso desse radical pode oxidar biomoléculas ou reagir com óxido nítrico, gerando peroxinitrito. Este, por sua vez, é mais reativo e pode modificar proteínas formando nitrotirosina. A nitrotirosina é reconhecida como marcador indireto de estresse oxidativo (Ceriello, 2002).

Choudhary e colaboradores (Choudhary, Chandra & Kale, 1997) demonstraram que o MG inibe a atividade da SOD *in vivo* em *in vitro*. Considerando a similaridade nos mecanismos de reação, o GA pode ter atuado da mesma forma em nosso estudo. Uma inibição da SOD a ponto de acarretar em um aumento de superóxido pode levar à inibição da CAT por este radical (Kono & Fridovich, 1982). A inibição da CAT, por sua vez, leva a um aumento nos níveis de H_2O_2, podendo, na presença de metais de transição, gerar o radical hidroxil.

Interessantemente, dos três órgãos analisados, apenas o fígado apresentou um acúmulo de CML. Por ser ricamente vascularizado, o fígado pode apresentar um aporte maior de toxinas circulantes. Outra possibilidade é que o ambiente intracelular seja mais suscetível à formação de AGEs. A presença de cobre (Cu^{+2}) num meio de incubação com glicose acentua a formação de AGEs em colágeno do tipo I (Sajithlal, Chithra & Chandrakasan, 1998). Quando na presença de ferro (Fe^{+2}), esta reação também é acelerada (Xiao, Cai & Liu, 2007). Ocorre que as concentrações de Fe^{+2} são maiores no fígado em comparação com o rim. Além deste fato, a oxidação de lipídios também pode gerar CML (Fu *et al.*, 1996). Sendo o fígado um órgão central no metabolismo de lipídios, a abundância destas moléculas no órgão facilita a reação ocasional com agentes oxidantes. O fígado também age como órgão central na eliminação dos AGEs, através das células endoteliais e de Kupfer, em um processo de endocitose (Smedsrod *et al.*, 1997). Logo, os AGEs

presentes no fígado, ou parte deles, podem ser oriundos da circulação, tendo acumulado pela ineficiência no processo de eliminação.

As estruturas analisadas neste trabalho apresentam complicações particulares no DM. Quadros de hiperglicemia, bem como a resistência à insulina, são fatores de risco para o desenvolvimento da esteatose hepática (Lewis & Mohanty, 2010). A esteatose se caracteriza pelo acúmulo de ácidos graxos no fígado, um processo que pode progredir para cirrose e esteato-hepatite. Este processo patológico tem a participação de estresse oxidativo e a aplicação de terapias antioxidantes é bastante investigada. Em modelo animal de fibrose hepática, por exemplo, o uso do antioxidante N-acetilcisteína reduz o estresse oxidativo e a deposição de fibronectina. Ainda, os níveis de CML no plasma estão correlacionados com a severidade da cirrose (Yagmur *et al.*, 2006). Além disso, AGEs induzem a expressão de RAGE em células estreladas hepáticas, aumentando a produção de ERO através da enzima NADPH oxidase (Guimaraes *et al.*, 2010). Portanto, o aumento nos níveis de AGEs circulantes, bem como o estresse oxidativo, inerentes ao DM, contribuem para o desenvolvimento de complicações hepáticas.

Modelos animais de DM corroboram teorias que colocam o estresse oxidativo como componente das complicações cardiovasculares. Animais diabéticos apresentam aumento na carbonilação de proteínas e na peroxidação lipídica no coração (Atalay *et al.*, 2004; Gumieniczek, 2005; Shirpoor *et al.*, 2009). O quadro diabético compromete tanto a função sistólica como a diastólica, aumentando em até 5 vezes o risco de problemas no coração (Khavandi *et al.*, 2009). A utilização de aminoguanidina, um inibidor de AGEs e quelante de aldeídos como o GA, reestabelece parâmetros como volume diastólico final e capacidade de distensão na

sístole (Wu *et al.*, 2008). Logo, além da integridade celular e talvez por prejudicarem a mesma, os AGEs interferem nos parâmetros fisiológicos da função cardíaca.

A insuficiência renal é característica comum em diabéticos. O elevado fluxo de glicose promove eventos de estresse oxidativo e formação de AGEs. O acúmulo de dano renal leva à permeabilidade de albumina e acúmulo de matriz extracelular, aumentando a proteinúria (Soldatos & Cooper, 2008). Ratos diabéticos apresentam maior produção de radical superóxido no glomérulo renal, sendo este excesso atenuado pela inibição da interação entre AGEs e RAGE (Coughlan *et al.*, 2009). Pacientes diabéticos apresentam acúmulo de AGEs no rim (Daroux *et al.*, 2010), além de haver correlação entre os níveis plasmáticos de CML e doença renal crônica (Semba *et al.*, 2010).

Selvaraj e colegas (Selvaraj, Bobby & Sridhar, 2008) discutem a possibilidade de eventos de estresse oxidativo promoverem a glicação proteica. Sabendo-se que tratamentos antioxidantes diminuem as taxas de glicação, é suposto que a participação de ERO possa desempenhar um papel importante na formação de AGEs. Produtos da peroxidação lipídica e H_2O_2 aceleram a glicação da hemoglobina. Além disso, danos mitocondriais podem aumentar a produção de ERO e reduzir a produção de ATP, levando a um acúmulo de glicose que, por fim, facilita a formação de AGEs (Edeas *et al.*, 2009). Rosca e colaboradores (Rosca *et al.*, 2005) demonstraram em modelo animal que o DM altera a função mitocondrial, reduzindo a atividade da cadeia transportadora de elétrons e aumentando a produção de superóxido. Estas alterações são acompanhadas por um aumento de AGEs nas proteínas mitocondriais e são revertidas pelo tratamento com aminoguanidina. Desta forma, supõe-se que situações de estresse oxidativo ou

hiperglicemia podem dar início a um círculo vicioso com elevada produção de ERO e AGEs.

Então, conforme os resultados apresentados, mostramos que o GA induz dano oxidativo e modula enzimas antioxidantes no fígado, rim e coração de ratos Wistar. É difícil precisar se a modulação das enzimas é causa ou consequência do dano oxidativo observado, bem como definir se os efeitos observados se devem à ação direta do GA ou à intermediação de AGEs. Assim, mesmo curtos eventos de hiperglicemia e inflamação, capazes de elevar os níveis de GA, podem promover a oxidação de biomoléculas e a formação de AGEs. A consequência destes eventos pode ser o acúmulo de AGEs nos órgãos e o desenvolvimento de complicações como esteatose hepática, placas ateroscleróticas e falência renal. Portanto, a regulação da produção de aldeídos como o GA parece ser fundamental na prevenção de eventos deletérios.

5. CONCLUSÕES

A partir dos resultados obtidos neste trabalho podemos concluir que:

1) A exposição aguda ao glicolaldeído circulante, em concentrações estimadas como suprafisiológicas, induz aumento nos marcadores de estresse oxidativo – carbonilação de proteínas, peroxidação lipídica e oxidação de tióis – no rim, fígado e coração de ratos Wistar. De forma geral, o dano oxidativo persiste por até 24 horas após a injeção do aldeído.
2) A presença do GA na circulação foi capaz de modular a atividade das enzimas SOD, CAT, e GLO. No rim, as três enzimas apresentaram redução em sua atividade após 6 e 24 horas. No fígado também houve inibição, mas esta foi observada até 12 horas após a injeção. No coração o GA induziu um aumento na atividade de SOD e GLO em 6 horas, tendo retornado aos valores do grupo controle no tempo de 12 horas. A atividade da CAT também se encontrou diminuída após exposição ao GA, tendo esta modulação persistido por até 12 horas após a injeção. A inibição das enzimas favorece um aumento na concentração de ERO como $O_2^{\bullet-}$ e H_2O_2, podendo acarretar em um aumento de espécies mais reativas como peroxinitrito e o radical $OH^{\bullet}$.
3) Mesmo num curto período de exposição, o GA foi capaz de promover a formação de CML, um dos AGEs mais estudados e relacionados a patologias. A formação foi observada somente no fígado, sugerindo diferentes suscetibilidades nos órgãos estudados. Este dado sugere que a

formação de AGEs pode ocorrer posteriormente no desenvolvimento das complicações renais e cardiovasculares do DM.

4) Portanto, a exposição aguda ao GA foi capaz de induzir um estado pró-oxidante nos órgãos apresentados nesta dissertação: fígado, rim e coração. A modulação de enzimas antioxidantes foi acompanhada por um aumento nos níveis de marcadores de estresse oxidativo. É importante ressaltar que apesar do dano oxidativo, não observamos alterações funcionais nos órgãos. Isto pode ser devido ao curto período de exposição. Mostramos neste trabalho, pela primeira vez, que o GA presente na circulação é capaz de alterar parâmetros do estado redox celular, podendo comprometer a fisiologia orgânica e desempenhar, assim, um papel importante nas complicações observadas no DM. Ainda, este trabalho cria perspectivas para uma melhor investigação da influência de aldeídos como o GA em patologias como sepse, DM, mal de Alzheimer e o próprio processo de envelhecimento.

6. PERSPECTIVAS

O presente trabalho sugere um importante papel para aldeídos de cadeia curta nas complicações de diabetes, através da modulação do estado redox. Entretanto, os mecanismos pelos quais esta modulação se dá ainda precisam ser bastante investigados. São perspectivas de continuação deste trabalho:

1) Avaliar os efeitos do GA sobre parâmetros de estresse oxidativo sobre o sistema nervoso central, bem como investigar alterações comportamentais ou de humor promovidas pelo tratamento;
2) Determinar a influência do GA sobre o estado redox e a fisiologia do pâncreas, sendo este uma estrutura central na patologia diabética;
3) Investigar a influência do GA sobre o metabolismo da glicose, determinando de que formas este aldeído pode contribuir para a manutenção da hiperglicemia;
4) Investigar a função mitocondrial nos órgãos avaliados neste trabalho, buscando um melhor entendimento dos mecanismos de ação do GA e da formação de ERO;
5) Avaliar a participação do RAGE nos fenômenos observados neste trabalho, quantificando seu conteúdo e inibindo sua interação com AGEs através de tratamento com a forma solúvel deste receptor.

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8. ANEXOS

8.1 Normas do Journal of Biochemical and Molecular Toxicology

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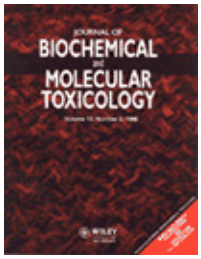

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General Instructions

Manuscripts should be written in English and should conform to the general style of the journal and specific instructions listed below. Although the Materials and Methods Section should be concise, sufficient experimental detail should be included to permit repetition. Do not include the same material in more than one section, e.g discussion in both Results and Discussion.

Form and Style

Type manuscripts with double spacing throughout and margins of at least 1 inch (25 mm). Indicate positions of tables and figures in the text using the left margin. Principal headings within the text should be typed in all capital letters and indented; subheadings should be typed, also indented, in upper and lower case letters; if a third level of heading is required, it should have a paragraph indent, underscoring, upper and lower case lettering, and, as with the primary and secondary headings, should be on a line separate from the text.

Full-Length Articles

These should include all of the following elements, each of which should start on a separate sheet of paper. They should not exceed the following size limits: Abstract, 150 words; Introduction, 550 words; Material and Methods - see General Instructions (above); Discussion - 550 words; Figures - 5; Tables - 5.

- Title page–title of article, author(s), affiliation(s) with complete addresses
- Word, figure and table counts: abstract; introduction; discussion, figures, tables
- Running title of not more than 40 characters, including spaces, and suggestions for key word index entries
- Mailing address of the person to whom proofs should be sent
- Abstract
- Introduction
- Materials and Methods
- Results
- Discussion
- Acknowledgments
- Footnotes
- References
- Figure legends
- Tables

Rapid Communications

These should not exceed four double-spaced pages, including tables and figures. The subdivision into Materials and Methods, Results, and Discussion can be omitted. A brief summary and a short title should be provided. These data of immediate importance to other researchers will be published rapidly.

Mini-reviews

Mini-reviews of timely importance will be published by invitation but submitted mini-reviews will also be considered. The manuscript should not exceed 15 typewritten pages and the subdivisions are at the discretion of the author(s).

Organization of the Manuscript

The *Abstract* should present the plan, rationale, and significant findings of the research. Do not use abbreviations in the abstract.

The *Introduction* should state the purpose of the investigation and its relation to work in the same field without extensive review of the literature.

Materials and Methods should be brief but adequate for repetition of the work. Refer to previous methods and describe only the pertinent modifications.

Results may be presented in tables or figure.

The *Discussion* should be concise, interpreting the data and relating it to other published works.

References should be cited in the text by a number in parentheses and listed at the end of the paper in order of citation in the text; i.e., in numerical sequence. The following are examples:

1. Bakry NMS, El-Rashidy AH, Eldefrawi AT, Eldefrawi ME. Direct actions of organophosphate anticholinesterases on nicotinic and muscarinic acetylcholine receptors. J. Biochem. Toxicol. 1988;3:235–259.

2. Tynes RE, Sabourin PJ, Hodgson E. Identification of distinct hepatic and pulmonary forms of microsomal flavin-containing monooxygenase in the mouse and rabbit. Biochem. Biophys. Res. Commun. 1985;126(3):1069–259.

3. DeBruin A. Biochemical Toxicology of Environmental Agents, 2nd repr. Amsterdam: Elsevier; 1980. 1544 p.

4. Neal RA. Microsomal metabolism of thionosulfur compounds: mechanisms and toxicological significance. In: Hodgson E, Bend JR, Philpot RM, editors. Reviews of Biochemical Toxicology. Amsterdam: Elsevier; 1980. p 131–172.

Include first and last page numbers. Accepted papers are cited as above indicating in press with the projected publishing date. For journal abbreviations, refer to the *Chemical Abstracts Service Source Index* (1969) and its supplements.

For the preparation of index entries for your article select *Key Words* under which your article should be indexed. Use the title and abstract of the article being indexed, considering carefully the words that are chosen for index listings. Limit your choices to those words which best describe the most significant findings in the paper. Avoid vague terms or those encompassing too large an area. Do not use abbreviations unless they are in general use (e.g., DDT, DNA, etc.).

Preparation of Tables

Tables should be numbered with Arabic numerals and cited consecutively in the text. Each table should be titled and typed double-spaced on a separate sheet. Sufficient experimental details (if not described in the text) should be included as a footnote using sequential lower case letters (as superscripts). Units must be indicated clearly for each of the entries in the table. Each column should carry an appropriate heading.

Define all statistical measures.

Complex tables may be submitted as "camera ready copy" for precise publication. Such tables should be typed single-spaced. Keep to a minimum the space between columns of figures. Horizontal rules above and below the column headings and at the end of the table body (i.e., before any table footnotes) should be added to the full width of the table; straddle rules should be drawn to the width of all the subcolumn heads which are below a major column heading. Do not add vertical rules in tables.

Multipliers by powers of 10 are sometimes useful. To minimize the confusion, employ decimal units (mM, nm, µL, ng, etc.) instead of exponent multipliers.

Figures

Figures must be submitted in a form suitable for reproduction with proportionate-sized lettering throughout. The maximum final printed page width in the journal is 7 inches (178 mm). If drawing to scale is not possible, please bear in mind that figures will be reduced to fit and lettering sizes should thus be scaled accordingly (a final depth of approximately 1/8", 3 mm is a good guide for lettering after reduction). Glossy black-and-white photographic prints must be supplied for all illustrations; do not send original artwork or films. Each figure must be marked clearly on the reverse (preferably on an adhesive label so that writing does not penetrate the photographic paper) with the author's name, figure number (arabic), and top of the figure. Do not mount the figures or use clips of any kind which will mark them. If multiple-part figures are used, each part must be sized proportionately to the other parts. Stereospecific illustrations must be submitted exactly as they will appear in the journal. Simple curves, e.g., dose-response, enzyme kinetics, should be reduced to a smaller size unless these are really complex. Spectra shoud be presented on a scale; that is, linear in frequency (or wave number) rather than in wavelength. In such cases indicate wavelength on a nonlinear scale at the top of the diagram (use nanometers, nm).

Figure titles and legends (containing definitions of all symbols used in the figure) should be typed double-spaced in list form on separate sheets and numbered consecutively in the sequence in which the figures are cited in the text.

Computer-generated laser-printed figures may be acceptable if printed on good quality paper and of sufficient clarity and contrast for subsequent photographic reduction.

All color figures will be reproduced in full color in the online edition of the journal at no cost to authors. Authors are requested to pay the cost of reproducing color figures in print. Authors are encouraged to submit color illustrations that highlight the text and convey essential scientific information. For best reproduction, bright, clear colors should be used. Dark colors against a dark background do not reproduce well; please place your color images against a white background wherever possible.

Chemical Formulas

Chemical formulas should be described in the text. Structural formulas, metabolic pathways and equations, and mathematical formulas should be presented for direct photographic reproductions (a stencil is suggested, or the use of transfer lettering – Chartpak, Letraset brands, for example – for a professional appearance). Computer-generated formulas printed with a laser printer may be acceptable if they are of sufficient clarity and contrast for subsequent photographic reduction.

Ionic charges should be designated as superscript, e.g., Ca^{2}, Mg^{2}, Na^{2}. The symbol for the isotope introduced is placed in square brackets, e.g., [14-C]-aldrin, [L- *methyl* -14-C]-methionine, [*cis* -14C]-chlordane. The symbol U indicates uniform and G general labeling, e.g., [U-14-C]-glucose or [G-14-C]-glucose (where the radioactivity is not uniformly distributed on all six carbons). The symbol indicating the configuration should precede the symbol for the isotope, e.g. [*cis* -14-C]-chlordane, D-[14-C]-glucose. The abbreviation 32-P may be used for radioactive inoganic phosphate. [Refer to *Biochem* . J. 169:1 (1978).]

For spectrophotometric data the molar absorption coefficient should be nm^{-1} cm^{-1}.

The composition of solutions and buffers should be specified in sufficient detail to indicate the concentrations of each species.

Abbreviations

Refer to *J. Biol. Chem.* 228, 6 (1963) or Abbreviations and Symbols for Chemical Names of Special Interest in Biological Chemistry; Revised Tentative Rules, **115** , 1 (1965). Also see below.

Avoid abbreviations in titles and summaries, because these are most often translated into other languages and are used by abstractors. Do not start sentences with abbreviations. Abbreviations and symbols should be used sparingly in the text and only if advantage to the reader results. They should be defined on first occurrence, with the abbreviation following in parentheses. Chemical equations, which traditionally depend on symbols, may utilize an abbreviation or symbol for a term which appears in full in the accompanying text. Trivial names are usually sufficiently short to eliminate any need to abbreviate.

For chemical terms, follow the usages given in the indexes of Chemical Abstracts [(1962) *Chem. Abst* . **56** ,IN-98N]. All abbreviations, symbols, and trivial *Collected Tentative Rules and Recommendations of the Commission on Biochmical Nomenclature* (IUPAC-IUB) (1973) are acceptable, as are those found in the following:

For stereochemistry (IUPAC Tentative Rule) (1970) *J. Org. Chem* . **35** , 28492867. For immunoglobulins (WHO document) (1972) Biochemistry 11, 3311–3312.

Enzymes

Where one or more enzymes figure prominently in a manuscript, authors should use the Recommended (Trivial) Name given by the *IUB Commission on Enzyme Nomenclature: Recommendations* (1972) of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (1973, Elsevier, Amsterdam). In some cases the Systematic Name or the reaction catalyzed should also be included.

Prefixes to the Names of Units

tera	10^{12}	T
giga	10^{9}	G
mega	10^{6}	M
kilo	10^{3}	k
centi	10^{-2}	c
milli	10^{-3}	m
micro	10^{-6}	μ
nano	10^{-9}	n
pico	10^{-12}	p
femto	10^{-15}	f
atto	10^{-18}	a

Units of Concentration

molar (mole/liter)	M
millimolar (mmole/liter)	mM (rather than 10^{-3} M)
micromolar (μmole/liter)	μM (rather than 10^{-6} M)
nanomolar	nM (not mμM)
picomolar	pM (not μμM)
part per million	mg/L, mg/kg, ppm
percent	% (w/v, v/v, w/w)

Other Words

logarithm (Briggsian)	log
logarithm (natural)	ln
standard deviation of series	S.D.
standard error of mean	S.E.

Physical and Chemical Properties

meter	m
centimeter	cm
millimeter	mm
micrometer (not micron)	μm (not μ)
nanometer	nm
picometer	pm
Angstrom (0.1 nm)	Å

square centimeter	cm^2
cubic centimeter	cm^3
liter	L (in tables only)
milliliter	mL
microliter	µL (not lambda)
milligram/cm^2	mg/cm^2
gram	g
milligram	mg
microgram	µg (not gamma)
second	s
minute	min
counts per minute	cpm
revolutions per minute	rpm
Curie	Ci
equivalent	eq
Svedberg unit of sedimentation coefficient (10^{-13} S)	S
mole	mol
cycle per second (Hertz)	Hz
retardation factor	R_F
acceleration of gravity	g
specific rotation	$\|a\|^t_{lambda}$
sedimentation coefficient	s
sedimentation coefficient in water at 20º, extrapolated to zero concentration	$S_{20,w}$
diffusion coefficient (usually given in $cm^2 S^{-1}$)	D
degree Centigrade or Celsius	ºC
degree absolute (Kelvin)	K
equilibrium constant	K
Michaelis constant	K_m
calorie	cal
kilocalorie	Cal
joule	J
gauss	G
lethal dose/concentation-50	LD-50 or LC-50

Footnotes in the text should be identified by superscript Arabic numerals and shoud be typed on a separate sheet; footnotes in the tables should be identified with superscript lower-case letters and placed at the bottom of the tables.

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