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INHIBITION OF GLUTATHIONE REDUCTASE BY ISOPROTERENOL OXIDATION PRODUCTS

FERNANDO REMIÃO*, HELENA CARMO, FÉLIX D. CARVALHO and MARIA L. BASTOS

ICETA/CEQUP, Toxicology Department, Faculty of Pharmacy, Rua Aníbal Cunha, 164, 4050 Porto, Portugal

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Oxidative stress induced by catecholamines is a well recognized toxic event. This effect has been extensively observed in the heart, where high levels of catecholamines cause enzyme inhibition, lipid peroxidation, energy depletion and myocardial necrosis. Catecholamines can be converted into *o*-quinones and undergo cyclization into aminochromes. This process can occur enzymatically or through autoxidation and involves the formation of free radicals. Aminochromes are highly reactive molecules that can cause oxidation of protein sulfhydryl groups and deamination catalysis, among other deleterious effects; in addition, inhibition of some enzymes has been also reported.

We have studied the effects of isoproterenol oxidation products (IOP) on glutathione reductase (GR) activity *in vitro*. Isoproterenol (ISO) autoxidation was conducted at 37°C in the dark, for 4 h at pH 7.0 and this process was monitored by UV spectrophotometry at both 340 and 490 nm. Addition of the autoxidized solution to GR in the presence of oxidized glutathione (GSSG) and NADPH showed that IOP inhibits GR in a competitive mode and that this effect increases during the 4 h incubation period. This inhibitory effect of IOP was partially prevented by the addition of reduced glutathione (GSH), L-cysteine and ascorbic acid to the reaction mixtures.

Keywords: Glutathione reductase; Catecholamines; Oxidative stress; Aminochromes; Isoproterenol; Antioxidants

Abbreviations: ISO, isoproterenol; IOP, isoproterenol oxidation products; GR, glutathione reductase; abs, absorbance; GSH, reduced glutathione; GSSG, oxidized glutathione

* Corresponding author. Tel.: 351-2-2078908. Fax: 351-2-2003977.
E-mail: remiao@mail.ff.up.pt.

INTRODUCTION

Glutathione in its reduced (GSH) and oxidized (GSSG) forms is the major thiol redox system of the cell, providing protection against oxidative stress. The GSH/GSSG balance in the cell is an important parameter, since it gives a good indication of the thiol oxidative status.[1] During conditions of optimal metabolism, the *in vivo* ratio of reduced GSH to GSSG can exceed 100 : 1.[2] To maintain a high cellular GSH/GSSG ratio, glutathione reductase (GR) (EC 1.6.4.2), a dimeric FAD-containing enzyme with a redox active disulphide at its active site, catalyses the NADPH-dependent reduction of GSSG to GSH:

$$GSSG + NADPH + H^+ \overset{GR}{\rightleftharpoons} 2GSH + NADP^+ \quad (1)$$

In this redox reaction the enzyme cycles between reduced and oxidized states, involving reversible disulphide bond formation.[3] GR is known to be sensitive to chemical modification of its active thiol groups.[3] Thus, the activity of GR can be modulated by the redox conditions of the reaction environment. Under extreme oxidizing conditions aggregates of GR may be formed which decrease GR activity.[4] In addition, incubation of GR in the presence of high concentrations of NADPH or NADH produces an almost complete inactivation of the enzyme.[5]

The β-agonist isoproterenol (isoprenaline, ISO) is a synthetic catecholamine which can be used in the treatment of asthma and shock,[6,7] and in emergencies to stimulate heart rate in patients with bradycardia or heart block.[6,7] Nevertheless, it has been replaced by other sympathomimetic drugs for these purposes. Notwithstanding, ISO can be related to the natural occurring catecholamines epinephrine, norepinephrine and dopamine. Epinephrine and dopamine also have therapeutic use.[6,7] Therefore, ISO is useful in experimental toxicology as a model compound for the study of catecholamine toxicity.[8–12] At high doses, catecholamines produce disturbance of heart function and metabolism as well as myocardial necrosis.[9] In fact, several myocardial toxic effects related to high levels of circulating ISO have been reported. These include alterations in calcium homeostasis resulting in extensive myocardial necrosis,[10] lipid peroxidation,[8,11] and decrease in ATP and creatine phosphate stores.[9] A decrease in the activity of superoxide dismutase, catalase, GR and glutathione-S-transferase in myocardial mitochondria has also been reported.[12] Although it is generally believed that excess catecholamines exert cardiotoxic effects primarily via

binding to adrenoceptors and causing intracellular calcium overload, oxidative stress is, more probably, one of the main mechanisms through which catecholamines exert their toxic effects. Spontaneous oxidation of catecholamines results in the formation of catecholamine-*o*-quinones, which generate aminochromes through cyclization.[9] Adrenochrome (which results from the cyclization of epinephrine-*o*-quinone) can be oxidized to several other compounds such as adrenolutin, 5,6-dihydroxy-1-methylindole (DHMI) or adrenochrome–adrenolutin dimer.[9] All these redox reactions generate free radicals. Consequently, catecholamine-*o*-quinones, aminochromes and the radical species resulting from the oxidation of catecholamines are thought to be involved in catecholamine-related toxicity.[11] Adrenochrome and oxidized ISO have been reported to produce cell damage and contractile failure in the isolated perfused rat heart.[9] It has also been reported that aminochromes oxidize protein sulfhydryl groups, catalyse the deamination of a variety of amines and amino acids, induce uncoupling of the mitochondria and inhibit monoamine oxidase and alkaline phosphatase.[9] A study conducted by Bironaite *et al.*,[13] shows that certain quinones, including some which are naturally occurring, may act as potent inhibitors of GR.

The occurrence of intracellular catecholamine oxidation does not always induce toxicity because the cell has multiple protective agents that limit free radical damage, namely free cysteine and GSH which are very important nucleophiles within the cell.[14] Ascorbate is another protective compound which is thought to exert direct antioxidant effects by acting as a one-electron reductant for many biologically relevant oxidants.[15] Thus, GSH, L-cysteine and ascorbic acid play essential roles in the defence against oxidative stress generated by free radicals. Considering that IOP may inhibit GR activity through an oxidative stress mechanism it would be expected that these antioxidants would prevent this effect. The aim of the present study was to evaluate GR activity in the presence of IOP and the influence of the antioxidants GSH, L-cysteine and ascorbic acid on the observed effects.

MATERIALS AND METHODS

Chemicals

L-Ascorbic acid, L-cysteine hydrochloride monohydrate, (±)-isoproterenol hydrochloride, β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH) tetrasodium salt, glutathione oxidized form (GSSG)

disodium salt, glutathione reduced form (GSH), ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate and glutathione reductase (Type VII, from bovine intestinal mucosa, EC 1.6.4.2) were obtained from Sigma Chemical Company (Madrid, Spain). Potassium dihydrogen phosphate (KH_2PO_4) was obtained from Merck (Lisbon, Portugal). All the chemicals were of analytical grade. The water used was deionized and tridistilled.

L-ascorbic acid, β-NADPH and L-cysteine were prepared in 0.2 M KH_2PO_4 and 2 mM EDTA (pH 7.0) buffer. ISO was prepared in 0.2 M KH_2PO_4 (pH 7.0) buffer.

The racemic form of isoproterenol was used as it is included as such in some medicinal formulae.

GR Stock Solution

The pure enzyme was stored at −20°C, at a concentration of 0.15 U/mL in 0.2 M KH_2PO_4 buffer (pH 7.0) containing 2 mM EDTA.

ISO Oxidation

An aqueous solution of ISO, 3 mM, was left to oxidize in 0.2 M KH_2PO_4 buffer (pH 7.0) saturated with carbogen at 37°C in the dark for a maximum period of 4 h. During this time, the oxidation of 1 : 3 buffer diluted ISO solution 1 mM ISO (final concentration) was monitored by spectrophotometry at 340 and 490 nm in a 96 well plate reader (Ceres 9000).

Spectra of ISO and IOP were obtained over the 200–800 nm wavelength range (Shimadzu UV-160).

^{1}HNMR spectra were taken on a Bruker AMX 300 instrument operating at 300.13 MHz, at ambient temperature, using a coaxial tube with deuterated water, which also served as reference. At the same time, it was made the saturation of the water signal.

GR Assay

The activity of GR was evaluated by following the oxidation of NADPH consumed during the reduction of GSSG at 340 nm, at a constant temperature of 30°C, using a 96 well plate reader (Ceres 9000). The incubation mixture contained 0.025 units of GR, 0.1 mM GSSG and 1 mM EDTA in 0.17 M KH_2PO_4 (pH 7.0) buffer. After 2 min pre-incubation, the reaction was initiated by the addition of NADPH (final concentration 0.1 mM). The

final volume of the assay mixture was 300 μL. The kinetics of the reaction were monitored for 5 min at 20 s intervals. A blank assay containing all components of the reaction mixture except the enzyme was performed to evaluate the non-enzymatic oxidation of NADPH and this value was subtracted from the assay values. Seven determinations were performed for all experimental conditions.

The effect of IOP on the inhibition of GSH reductase was evaluated by performing the assay as described above except that the product of ISO oxidation was added to the reaction mixture (and diluted 1 : 3), just prior to the 2 min incubation. Control assays were performed in the absence of IOP.

The effects of GSH (0.01–2 mM), L-cysteine (0.01–2 mM) and ascorbic acid (0.01–80 mM) on the IOP-mediated inhibition of GSH reductase were studied by their addition to the reaction mixture just before IOP addition.

The mode of inhibition by IOP was investigated by conducting the assay at different concentrations of GSSG (0.1–1 mM). Double-reciprocal plots of enzymatic velocity versus substrate (GSSG) concentration were used to assess the nature of the interaction between GR and ISO oxidation products.

Statistical Analysis

Data were analysed by two-way analysis of variance (ANOVA), followed by the Duncan Test. All data are expressed as mean ± standard deviation (M ± SD).

RESULTS

The oxidation of ISO was monitored every hour for 4 h by using UV spectroscopy. The ISO spectrum (KH_2PO_4 buffer, pH 7.0) shows maximum absorption at 279 nm. During the 4 h period, the maximum absorption at 279 nm remained but the absorption levels increased between 300 and 650 nm (Figure 1), a higher increase in absorbance being observed near 300 nm rather than at 650 nm. However, no new independent absorption maximum of the ISO oxidized solution was observed. Oxidation of the ISO solution was then monitored at 340 nm, the wavelength at which we monitored GR activity, and at 490 nm, the wavelength band at which aminochromes have been monitored by others.[16–19] The study was performed with only a single ISO concentration (pH, light, temperature, ionic strength, carbogen saturation and oxidation time variables always being controlled) and showed a variation in absorption at 340 and 490 nm with in the same

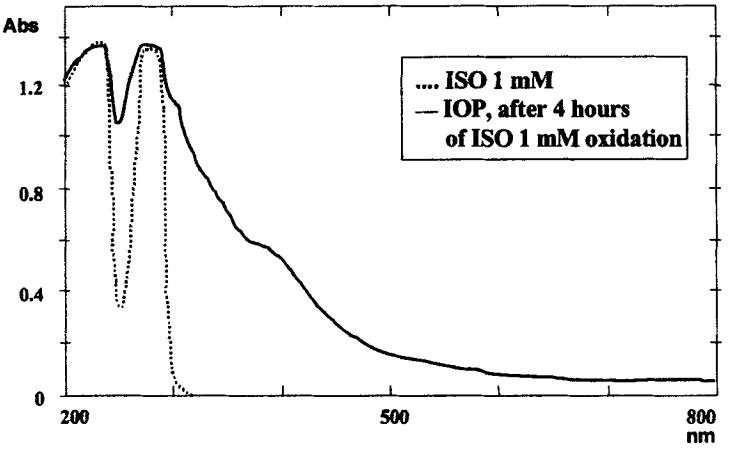

FIGURE 1 Absorbance spectrum of ISO (1 mM) and IOP solution after 4 h of ISO (1 mM) oxidation in 0.2 M KH_2PO_4 buffer (pH 7.0) saturated with carbogen, at 37°C and in the dark.

oxidation time for different ISO solutions (data not shown). Monitoring of the ISO oxidation reaction during a 4 h period by NMR was not possible because most of the ISO remained unoxidized and interfered with the spectra.

GR activity was determined after treatment with ISO oxidized to different extents. Figure 2 shows the extent of GR inhibition versus IOP absorption levels at 340 and 490 nm obtained with the same ISO solution at different oxidation times. A high polynomial correlation (order 2) ($R^2 = 0.9644$ for 340 nm and $R^2 = 0.9842$ for 490 nm) was obtained between the increase in GR inhibition and the increase in IOP absorption levels (Figure 2). On the other hand, only a poor polynomial correlation (order 2) ($R^2 = 0.212$ for 340 nm and $R^2 = 0.2613$ for 490 nm) was obtained between absorbance at 340 or 490 nm and extent of GR inhibition in the presence of IOP, obtained with different ISO oxidized solutions at different extents of oxidation (Figure 3). An assay was performed with a freshly prepared ISO solution and no enzymatic inhibition was detected (data not shown). The smallest extent of GR inhibition was $8.5 \pm 4.4\%$ of the control value, obtained with ISO oxidized solutions with low absorbances (abs. 0.024 at 340 nm; abs. 0.003 at 490 nm) after only 15 min of ISO oxidation. The greatest extent of GR obtained was 64.5% of the control value after 4 h of ISO oxidation (abs. 0.558 at 340 nm; abs. 0.077 at 490 nm).

In order to investigate the mode of the GR inhibition by IOP, a double-reciprocal plot of $1/V_0$ versus 1/[GSSG] was performed at different levels of ISO oxidation and a control assay (Figure 4). There was no difference in the

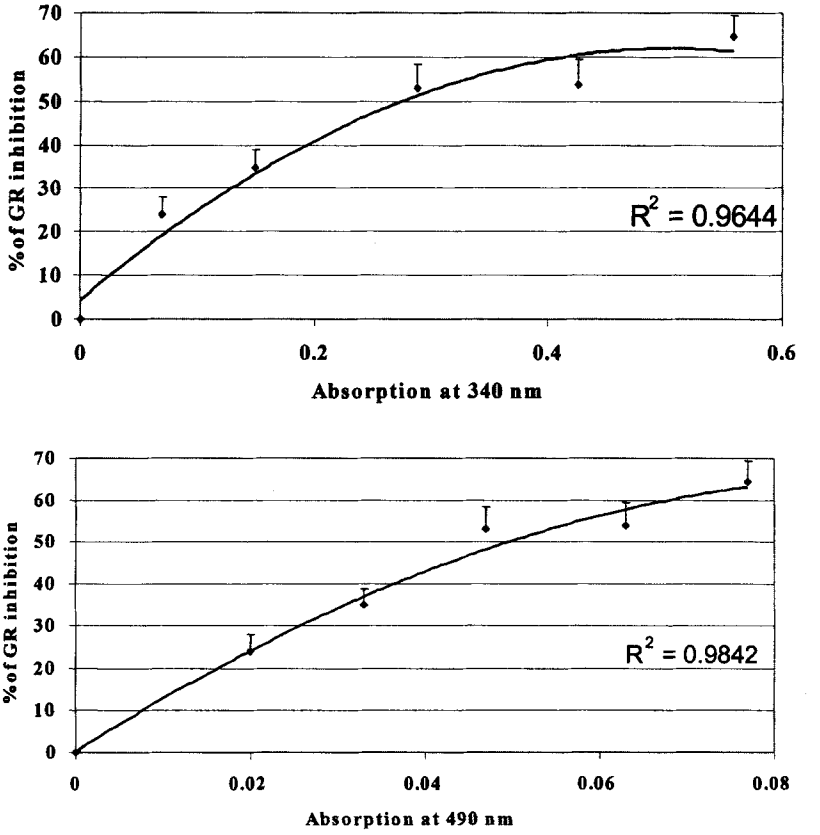

FIGURE 2 Rate of IOP-induced GR inhibition versus IOP absorption at 340 and 490 nm obtained with the same ISO solution at different oxidation times. Each data point is the M ± SD of 7 determinations.

maximum velocity (V_{max}) for GR, but the Michaelis–Menten constant (K_m) increased with increasing ISO oxidized solution absorbance. These results suggest that the inhibition was competitive with respect to GSSG.

The protective effect of three different antioxidants, GSH, L-cysteine and ascorbic acid against the IOP-induced GR inactivation was investigated. Figure 5 shows the effect of GSH concentration (0.01, 0.1, 1.0, 1.5 and 2.0 mM) on GR activity in the presence and absence of IOP. As expected, GSH by itself reduced GR activity at concentrations of 1.0, 1.5 and 2.0 mM [GR activity: 84.8 ± 8.9% ($p \leq 0.001$), 75.9 ± 9.3% ($p \leq 0.001$) and 68.3 ± 8.6% ($p \leq 0.001$) of control values, respectively]. However, in an assay where IOP (abs. 0.498 at 340 nm; abs. 0.060 at 490 nm) reduced GR activity to 55.6 ± 1.7% ($p \leq 0.001$), although 0.01 and 2.0 mM of GSH did not

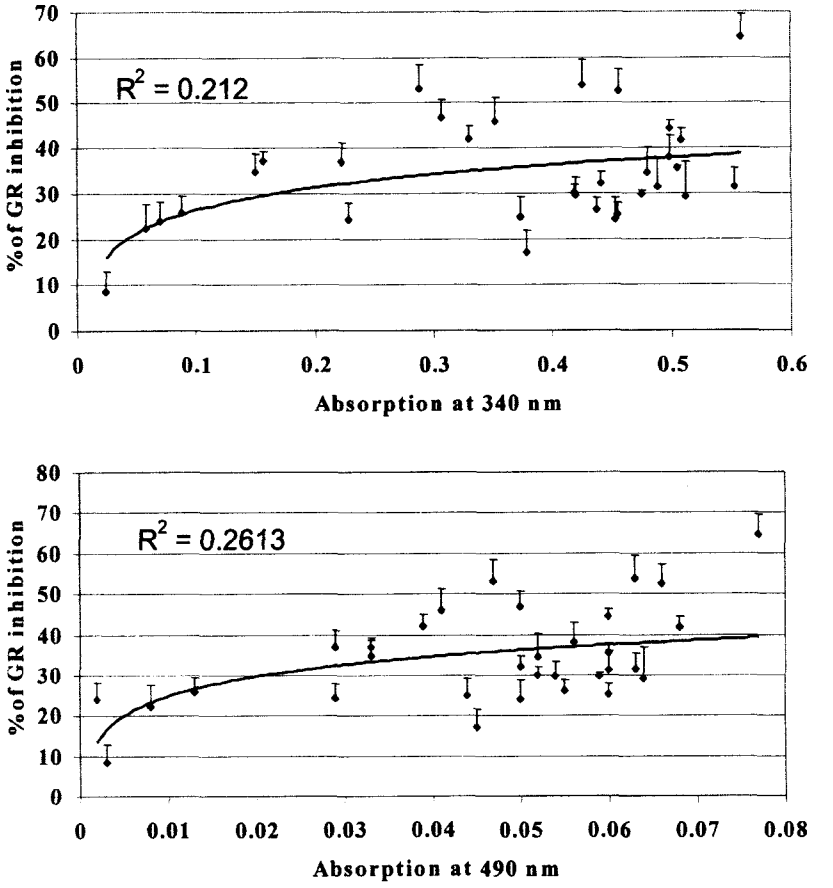

FIGURE 3 Rate of IOP-induced GR inhibition versus IOP absorption at 340 and 490 nm obtained with several ISO solutions at different oxidation times. Each data point is the M ± SD of 7 determinations.

affect significantly the GR activity, concentrations of 0.1, 1.0 and 1.5 mM of GSH enhanced GR activity to 65.0 ± 1.6% ($p \leq 0.05$), 66.0 ± 2.4% ($p \leq 0.01$) and 64.2 ± 1.8% ($p \leq 0.05$), respectively. Nevertheless, all these three GR activities were still significantly lower than those obtained with the same concentrations of GSH but in the absence of IOP.

In Figure 6 the effect of L-cysteine on GR activity and on IOP-induced GR inhibition is presented. L-cysteine by itself reduced GR activity at all the studied concentrations: 0.01, 0.05, 0.1, 0.5 and 1.0 mM [GR activity: 91.7 ± 13.7% ($p \leq 0.05$), 82.9 ± 7.9% ($p \leq 0.001$), 87.0 ± 9.9% ($p \leq 0.01$), 80.7 ± 7.3% ($p \leq 0.001$) and 75.2 ± 8.8% ($p \leq 0.001$) respectively]. However, in an assay where IOP (abs. 0.498 at 340 nm; abs. 0.056 at 490 nm)

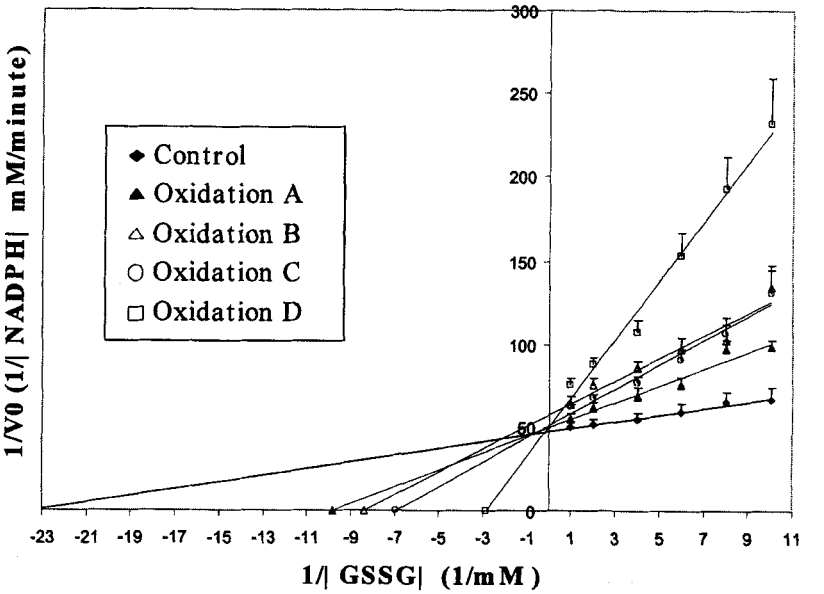

FIGURE 4 Double-reciprocal plots of $1/V_0$ versus 1/[GSSG] at 4 different ISO oxidation levels and control. Oxidation A: abs. 0.153 at 340 nm and 0.033 at 490 nm; Oxidation B: abs. 0.298 at 340 nm and 0.049 at 490 nm; Oxidation C: abs. 0.441 at 340 nm and 0.065 at 490 nm and Oxidation D: abs. 0.569 at 340 nm and 0.078 at 490 nm. Each data point is the M ± SD of 14 determinations.

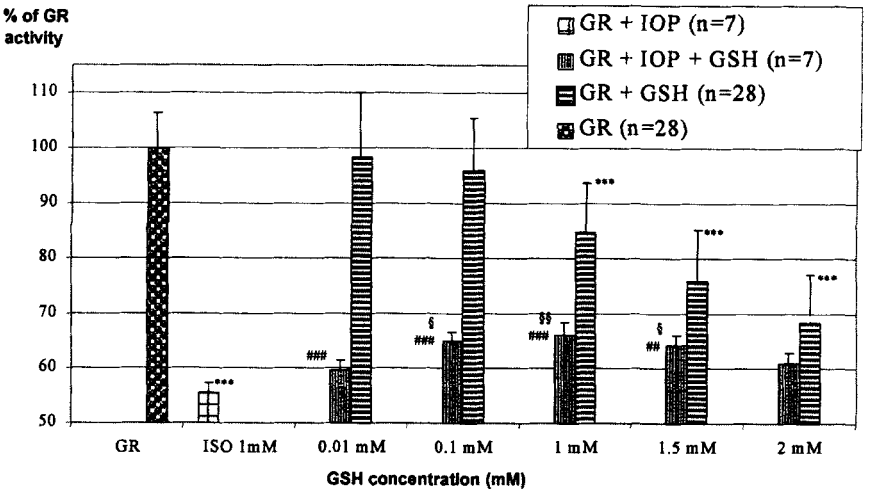

FIGURE 5 GSH effect on GR activity and on IOP-induced GR inhibition. The IOP absorption at 340 nm was 0.498 and at 490 nm was 0.060. The symbols *, § and # indicate the significance level. One symbol: $p \leq 0.05$; two symbols: $p \leq 0.01$; three symbols: $p \leq 0.001$. * compares GR + IOP and GR + GSH with GR. § compares GR + IOP + GSH with GR + IOP. # compares GR + IOP + GSH with GR + GSH. Data are expressed as M ± SD.

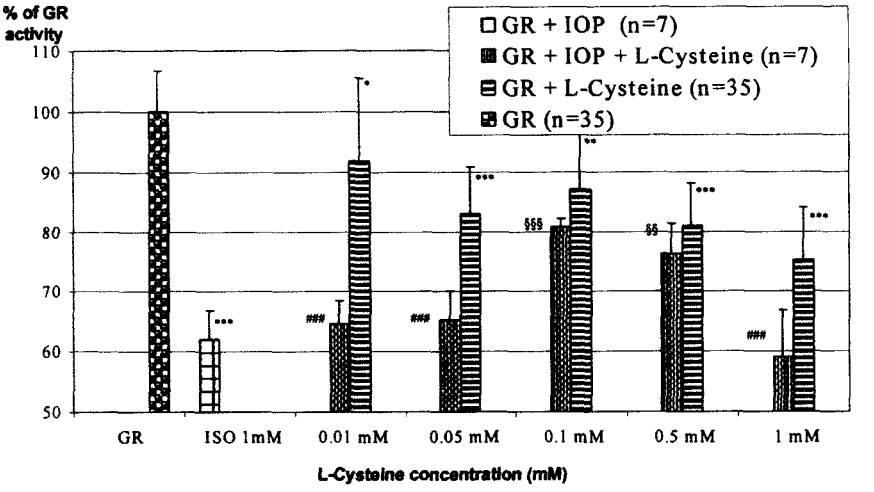

FIGURE 6 L-cysteine effect on GR activity and on IOP-induced GR inhibition. The IOP absorption at 340 nm was 0.498 and at 490 nm was 0.056. The symbols *, § and # indicate the significance level. One symbol: $p \leq 0.05$; two symbols: $p \leq 0.01$; three symbols: $p \leq 0.001$. * compares GR + IOP and GR + L-cysteine with GR. § compares GR + IOP + L-cysteine with GR + IOP. # compares GR + IOP + L-cysteine with GR + L-cysteine. Data are expressed as M ± SD.

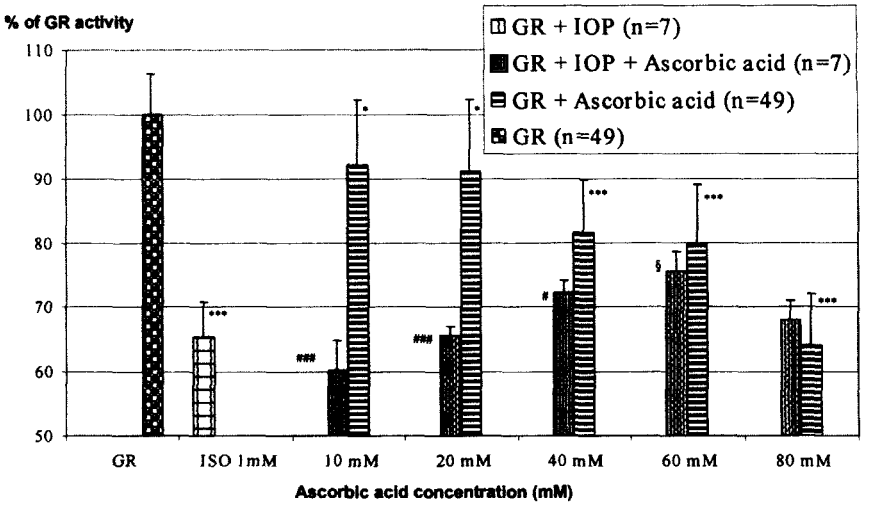

FIGURE 7 Effect of ascorbic acid on GR activity and on IOP-induced GR inhibition. The IOP absorption at 340 nm was 0.498 and at 490 nm was 0.056. The symbols *, § and # indicate the significance level. One symbol: $p \leq 0.05$; two symbols: $p \leq 0.01$; three symbols: $p \leq 0.001$. * compares GR + IOP and GR + Ascorbic acid with GR. § compares GR + IOP + Ascorbic acid with GR + IOP. # compares GR + IOP + Ascorbic acid with GR + Ascorbic acid. Data are expressed as M ± SD.

reduced GR activity to 62.0 ± 4.7% ($p \leq 0.001$), although 0.01, 0.05 and 1.0 mM of L-cysteine did not affect significantly the GR activity, concentrations of 0.1 and 0.5 mM of L-cysteine enhanced the GR activity to 80.6 ± 1.5% ($p \leq 0.001$) and 76.1 ± 5.1% ($p \leq 0.01$), respectively. In contrast to that observed with GSH, the activity of GR in the simultaneous presence of IOP and 0.1 or 0.5 mM of L-cysteine was not significantly different from GR activity in the presence of the same L-cysteine concentration but in the absence of IOP.

The results of the same assay conducted with ascorbic acid are shown in Figure 7. In this assay, ascorbic acid reduced GR activity at all the concentrations studied: 10, 20, 40, 60 and 80 mM [GR activity: 92.1 ± 10.2% ($p \leq 0.05$), 91.1 ± 11.2% ($p \leq 0.05$), 81.4 ± 8.4% ($p \leq 0.001$), 79.7 ± 9.2% ($p \leq 0.001$) and 64.2 ± 7.9% ($p \leq 0.001$) respectively]. In the presence of IOP (abs. 0.480 at 340 nm; abs. 0.052 at 490 nm), although 10, 20, 40 and 80 mM of ascorbic acid did not affect significantly the GR activity, a concentration of 60 mM of ascorbic acid enhanced GR activity from 65.4 ± 5.5% to 75.5 ± 3.1% ($p \leq 0.05$), which is not significantly different from GR activity in the presence of 60 mM of ascorbic acid only.

DISCUSSION

Solutions of ISO at pH 7.0 and 37°C rapidly become coloured red due to oxidation of ISO to aminochrome (Figure 8). In fact isoproterenol as well as epinephrine, norepinephrine and dopamine oxidize to the catecholamine-*o*-quinone. According to Hawley *et al.*[20] the rate of internal cyclization of catecholamine-*o*-quinone to respective aminochrome varies for the different catecholamines. It seems that dopamine has the lowest internal cyclization rate, while isoproterenol has a six-fold lower internal cyclization rate when compared to epinephrine, although 20 times higher than norepinephrine.[20] The aminochrome can also undergo further oxidation similarly to that shown for adrenochrome. In fact adrenochrome isomerizes to adrenolutin which can oxidize to 5,6-dihydroxy-1-methylisatin (DHMIs) or to adrenochrome–adrenolutin dimer.[8] All these oxidative reactions produce free radicals.[8] In our study a broad increase in the absorption levels was observed between 300 and 650 nm during oxidation of ISO solution (Figure 1). The higher increase in absorbance observed near 300 nm rather than 650 nm is most probably due to oxidation products of aminochrome in accordance with the study of Misra and Fridovich, who observed that oxidation of adrenochrome decreases its absorbance at 480 nm and increases

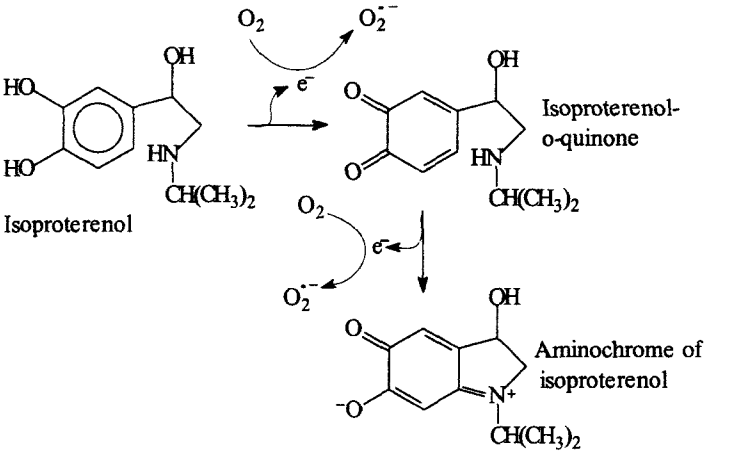

FIGURE 8 Proposed mechanism for oxidation of isoproterenol (Adapted from Rupp *et al.*[8] and Dhalla *et al.*[11]).

the respective absorbance at 300 nm.[16] The characteristic absorption maximum between 475 and 483 nm of aminochromes has been used for monitoring these compounds.[16,17] Dopaminochrome has been monitored by dual-wavelength spectrophotometry at 485 and 580 nm[18] or 305 and 475 nm.[19] In our study, ISO oxidation was monitored at 490 nm, and at 340 nm where GR activity was measured.

Although during the 4 h oxidation period the ISO absorption maximum at 279 nm was always seen in the spectrum, only ISO could be detected by NMR, which means that only a small amount of ISO undergoes oxidation; freshly prepared ISO solutions did not show any inhibitory effect on GR. Thus it was concluded that GR was inhibited by IOP and that this inhibition appeared to be dependent on ISO oxidation levels (Figures 2 and 3). These results are in agreement with a study conducted by Ithayarasi and Devi,[12] who reported a decrease in the activity of mitochondrial superoxide dismutase, catalase, glutathione peroxidase, glutathione-S-transferase and GR in the myocardium of ISO-treated rats. In the same study, α-tocopherol pre-treated rats administered ISO had antioxidant enzyme activities at near normal levels.[12] Further evidence of the importance of oxidative stress on ISO toxic effects is presented in a study by Ramos and Acosta[10] where a decrease in myocytes GSH content caused by toxic concentrations of ISO was shown. At the enzyme level, although to our knowledge no study with IOP has been previously performed, the inhibition of GR[13,21] and glutathione-S-transferase[22,23] by quinones, as well as monoamine oxidase,[24] alkaline phosphatase,[25] Type V adenylyl cyclase,[26] catechol-*O*-methyltransferase[27]

and heart sarcolemmal Na^+-K^+ ATPase[28] by different aminochromes have all been reported. GR activity is also inhibited by Fenton systems like $Cu(II)/H_2O_2$/catecholamine.[29] Thus, it seems likely that the inhibition of GR is due to isoproterenol-*o*-quinone, aminochrome, oxidation products of the aminochrome or reactive oxygen species generated during these oxidation reactions.

The data presented in Figure 2 result from an inhibition study that was conducted with the same ISO solution, but at different extents of oxidation. In this study, there is a good correlation between inhibitory effect and the absorption values at 340 and 490 nm. The polynomial curves (order 2) show a plateau at higher levels of oxidation, which could represent enzyme saturation. Data obtained from different extents of ISO oxidation obtained from various ISO solutions are shown in Figure 3. The correlation is poorer than that observed in the preceding study, since for similar absorption values at 340 and 490 nm different inhibitory effects are observed in the diverse ISO solutions. These results occur due to a broad spectrum of oxidized products resulting from ISO autoxidation, to the difficulty in controlling this process and to the fact that the wavelength monitored may not correspond to a maximum of absorption for IOP. Thus, the introduction of two variables, different times of ISO incubation and different concentrations or components of IOP, can explain the poor correlation obtained. The best correlation between inhibitory effect and absorbance was obtained at 490 nm. This observation and the fact that aminochromes have a maximum of absorption at this wavelength could indicate a direct involvement of the aminochrome resulting from ISO oxidation in GR inhibition. It is noteworthy that the rate of isoproterenol-*o*-quinone internal cyclization is six-fold lower than that of adrenaline[8] and that catecholamine-*o*-quinones have been shown to induce nucleophilic attacks on thiol groups of proteins.[8,11]

Although previous reports for Na^+-K^+ ATPase inhibition by adrenochrome or GR inhibition by quinones describe non-competitive inhibition,[28,13] the results shown in Figure 4 suggest that the IOP-induced GR inhibition is competitive with respect to GSSG. This fact is in accordance with a competitive inhibition to GSSG involving the disulphide active site in GR. The catalytic activity of GR requires two steps, the first is the reduction of the Cys58–Cys63 disulphide bridge and the second step involves the interaction of the reduced enzyme with GSSG to produce two molecules of GSH and the oxidized enzyme.[4,30] Thus, we studied the GR inhibition by IOP in the presence of two thiol antioxidants (GSH and L-cysteine) and a non-thiol antioxidant (ascorbic acid).

GSH and L-cysteine began to inhibit GR activity at a concentration of 1 and 0.01 mM, respectively. This inhibitory effect has also been reported by

other authors[6,31] and appears to be related to the high reducing conditions which can produce thiols at the active sites that can be oxidized by air to disulphides or interact with, and block, a site on a second enzyme molecule.[4,5] In the presence of IOP, GSH and L-cysteine enhanced GR activity. L-Cysteine appears to have the higher protective effect. It increases GR activity and this effect occurs at lower antioxidant concentrations. Additionally, GR activity, after inhibition by IOP, in the presence of 0.1 or 0.5 mM of L-cysteine was similar to the GR activity obtained with only the antioxidant. These results seem to indicate that the targets for the inhibitory effect of IOP are the sulfhydryl groups of cysteine residues in the GSSG active site of GR, which could be oxidized by IOP.

Ascorbic acid, which also had a GR inhibitory effect in concentrations higher than 10 mM, enhanced GR activity in the presence of IOP, but only at a 60 mM concentration. This concentration is very high when compared to the sulfhydryl antioxidants and is consistent with the importance of the thiol group at the enzyme active site.

The proposed mechanism for GR inhibition by IOP is the oxidation of sulfhydryl groups present at the GSSG enzyme active site, possibly mediated by aminochromes. The protein sulfhydryl oxidation by aminochromes as an enzyme inhibition mechanism has already been proposed by Dhalla *et al.*[9] for monoamine oxidase and alkaline phosphatase.

These results suggest that the inhibition of GR by IOP can increase the oxidative stress resulting from isoproterenol oxidation. In fact, the inhibition of GR reduces the capacity of the cell to restore the physiological levels of GSH, which may be depleted as a result of its antioxidant activity against free radicals and quinone products of ISO oxidation (Figure 8).

In conclusion, the present study shows that IOP inhibit GR activity. This inhibition appears to result from a partial ISO autoxidation and seems to increase with increasing ISO oxidation levels until enzyme saturation occurs. This inhibitory effect appears to be competitive with respect to GSSG and the GR sulfhydryl groups at the GSSG active site seem to be crucial to this effect. The inhibitory GR effect of IOP is a toxic effect, which may be related to catecholamine autoxidation.

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