BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2009), 158, 1932–1941

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Hydrogen peroxide affects contractile activity and anti-oxidant enzymes in rat uterus

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Background and purpose: The effects of hydrogen peroxide (H_2O_2) on uterine smooth muscle are not well studied. We have investigated the effect and the mechanism of action of exogenous hydrogen peroxide on rat uteri contractile activity [spontaneous and calcium ion (Ca^{2+})-induced] and the effect of such treatment on anti-oxidative enzyme activities.
Experimental approach: Uteri were isolated from virgin Wistar rats and suspended in an organ bath. Uteri were allowed to contract spontaneously or in the presence of Ca^{2+} (6 mM) and treated with H_2O_2 (2 μM–3 mM) over 2 h. Anti-oxidative enzyme activities (manganese superoxide dismutase-MnSOD, copper-zinc superoxide dismutase-CuZnSOD, catalase-CAT, glutathione peroxidase-GSHPx and glutathione reductase-GR) in H_2O_2-treated uteri were compared with those in uteri immediately frozen after isolation or undergoing spontaneous or Ca^{2+}-induced contractions, without treatment with H_2O_2. The effect of inhibitors (propranolol, methylene blue, L-NAME, tetraethylamonium, glibenclamide and 4-aminopyridine) on H_2O_2-mediated relaxation was explored.
Key results: H_2O_2 caused concentration-dependent relaxation of both spontaneous and Ca^{2+}-induced uterine contractions. After H_2O_2 treatment, GSHPx and MnSOD activities were increased, while CuZnSOD and GR (In Ca^{2+}-induced rat uteri) were decreased. N^{ω}-nitro-L-arginine methyl ester antagonized the effect of H_2O_2 on Ca^{2+}-induced contractions. H_2O_2-induced relaxation was not affected by propranolol, potentiated by methylene blue and antagonized by tetraethylamonium, 4-aminopyridine and glibenclamide, with the last compound being the least effective.
Conclusions and implications: H_2O_2 induced dose-dependent relaxation of isolated rat uteri mainly via changes in voltage-dependent potassium channels. Decreasing generation of reactive oxygen species by stimulation of anti-oxidative pathways may lead to new approaches to the management of dysfunctional uteri.
British Journal of Pharmacology (2009) **158,** 1932–1941; doi:10.1111/j.1476-5381.2009.00490.x; published online 16 November 2009

Keywords: rat uterus; H_2O_2; potassium channels; SOD

Abbreviations: 4-AP, 4-aminopyridine; CAT, catalase; CuZnSOD, copper-zinc superoxide dismutase; GR, glutathione reductase; GSHPx, glutathione peroxidase; K_v, voltage-gated K^+ channels; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MnSOD, manganese superoxide dismutase; NO^+, nitrosonium ion; NO^-, Nitroxyl ion; $O_2^{\bullet -}$, superoxide; ROS, reactive oxygen species; SOD, superoxide dismutase; TEA, tetraethylamonium

Introduction

Hydrogen peroxide (H_2O_2) is a key player in the metabolism of reactive oxygen species (ROS; Droge, 2001). H_2O_2 is an uncharged two-electron oxidant that has a long half-life in biological systems and is capable of diffusing across cell membranes. Furthermore, H_2O_2 is considered to be a cell-signalling molecule in its own right (Bergendi *et al.*, 1999). A complex set of enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx) and glutathione reductase (GR), together with low molecular weight compounds, form the anti-oxidative defence system that regulates the concentrations of ROS, within non-toxic homeostatic levels (Halliwell and Gutteridge, 2007). Under some conditions, ROS production can exceed anti-oxidative defence, resulting in overt oxidative stress. Such conditions include

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Received 20 March 2009; revised 16 July 2009; accepted 23 July 2009

muscle fatigue (Davies *et al.*, 1982), diabetes (Karasu, 2000), hypertension (Lacy and O'Connor, 1998) and particularly in the present context, intermittent ischemia induced by powerful myometrial contractions restricting blood flow to the uterus, causing a reperfusion/ischemia injury (Nakai *et al.*, 2000; Warren *et al.*, 2005).

The contribution of H_2O_2 to the regulation of myometrial contractility is not fully understood, and is still under investigation. Previous studies have shown that H_2O_2 can act on smooth muscles as both a contractile and a relaxing agent (Gao and Lee, 2005; Gil-Longo and Vazquez, 2005; Warren *et al.*, 2005). In some cases, H_2O_2 exhibits a biphasic effect (Gao *et al.*, 2003; Lucchesi *et al.*, 2005). Various steps during the molecular mechanisms underlying muscle contraction have been shown to be susceptible to redox modulation, such as the opening probability of sarcoplasmic reticulum Ca^{2+} release channels (Aghdasi *et al.*, 1997), Ca^{2+} reuptake by the sarcoplasmic reticulum (Andrade *et al.*, 1998) and myofibrilar Ca^{2+} sensitivity (Wilson *et al.*, 1991; Andrade *et al.*, 1998). H_2O_2-induced relaxation involves various mechanisms. H_2O_2 can mediate vasorelaxation depending on the vessel type and disease status (Ardanaz and Pagano, 2006), increase nitric oxide (NO) production through long-term up-regulation of endothelial NO synthase expression at the transcriptional level (Drummond *et al.*, 2000) and acute activation of the enzyme through increased post-translational modification (phosphorylation) (Thomas *et al.*, 2002). It has also been shown that H_2O_2 can directly promote relaxation by a cyclic guanosine monophosphate (cGMP)-dependent mechanism (Burke and Wolin, 1987; Yang *et al.*, 1999). Several studies have reported that H_2O_2 can mediate smooth muscle relaxation as an endothelium-derived hyperpolarization factor (EDHF) via activation of potassium ion (K^+) channels (Barlow *et al.*, 2000; Iida and Katusic, 2000; Gao *et al.*, 2003; Rogers *et al.*, 2007). K^+ channel opening leads to hyperpolarization and lowering of the Ca^{2+} concentration, resulting in smooth muscle relaxation. To date, several subtypes of K^+ channels have been identified in the myometrium. The most abundant and most well studied include large-conductance Ca^{2+}- and voltage-sensitive K^+ channels (BK_{Ca}), adenosine triphosphate (ATP)-sensitive K^+ channels (K_{ATP}), voltage-gated K^+ channels (K_v) and small-conductance Ca^{2+}-sensitive K^+ channels (SK; all nomenclature follows Alexander *et al.*, 2008). Various agents, including levcromakalim, pinacidil and nicorandil, that act by opening K^+ channels, have been used for the treatment of dysfunctional smooth muscle activity (Novakovic *et al.*, 2007).

Numerous studies have studied the effects of H_2O_2 on smooth muscle activity. The majority have explored its impact within blood vessels. Few have considered the role of H_2O_2 in myometrial smooth muscle activity, and above all, its role in the 'basal' uterine state (uninfluenced by dramatic changes in its hormonal status during pregnancy and parturition). Therefore, as changes in H_2O_2 concentration may contribute to the disruption of normal uterine contractile activity during the oestrous cycle, affecting production of other ROS and affecting changes in the concentration of Ca^{2+}, the aim of our study was to determine the effects of H_2O_2 on the myometrial activity of virgin Wistar rat uteri with respect to two types of activation: spontaneous and Ca^{2+}-induced, and to correlate these effects with changes in endogenous anti-oxidative defence. A range of inhibitors, N^{ω}-nitro-L-arginine methyl ester (L-NAME; NOS inhibitor), methylene blue (MB; cGMP signalling pathway inhibitor), propranolol (non-selective β-adrenoceptor antagonist), tetraethylamonium (TEA; non-selective K^+ channel inhibitor), glibenclamide (selective ATP dependent K^+ channel inhibitor) and 4-aminopyridine (4-AP; voltage-dependent K^+ channel inhibitor) were used in an attempt to identify the signalling pathways, used by H_2O_2 in this tissue.

Methods

Experimental system

All protocols for handling rats were approved by the local ethics committee for animal experimentation that strictly follows international regulations. Isolated uteri from virgin Wistar rats (200–250 g) in oestrus phase of the oestrous cycle, as determined by examination of a daily vaginal lavage (Marcondes *et al.*, 2002), were used.

Isolated organ bath studies

All rats were killed by cervical dislocation. The uterine horns were rapidly excised and carefully cleaned of surrounding connective tissue and mounted vertically in a 10 mL volume organ bath containing De Jalon's solution (see below for composition) aerated with 95% oxygen and 5% carbon dioxide at 37°C. The uteri, spontaneously active or contracting to Ca^{2+}- (6 mM), were allowed to equilibrate at 1 g tension before addition of the experimental drugs. H_2O_2 was added cumulatively at the following final concentrations: 2, 20, 200, 400 and 3 mM. Myometrial tension was recorded isometrically with a TSZ-04-E isolated organ bath and transducer (Experimetria, Budapest, Hungary). The same concentrations of H_2O_2 were added to isolated uteri that had been pre-incubated with various inhibitors. To determine the effects of an inhibitor alone on uterine contractility, it was added 15 min before adding H_2O_2. The following inhibitors were used: propranolol (1 μM), MB, (0.4 μM), L-NAME (10 μM), TEA (6 mM), glibenclamide (6 μM) and 4-AP (1 mM). Each concentration of H_2O_2 was left to act for 15 min. In experiments employing 4-AP, the highest dose of H_2O_2 (3 mM) was left to act for 30 min. Seven to 10 uteri were used per experiment. The number of uteri (*n*) for each experiment is given in the figure legends.

After treatment, the samples were immediately frozen using liquid nitrogen and then stored at –80°C until analysis.

Determination of anti-oxidative enzyme activities

Thawed uteri were homogenized and sonicated in 0.25 M sucrose, 1 mM ethylenediaminetetraacetic acid and 0.05 M Tris-HCl buffer pH 7.4 before centrifugation for 90 min at 105 000 × *g*. The supernatant was used to determine enzyme activities (using a Shimadzu UV-160 spectrophotometer, Shimadzu Scientific Instruments, Shimadzu Corporation, Kyoto,

Japan). Superoxide dismutase (SOD) activities were determined by the adrenaline method (Misra and Fridovich, 1972). One unit of activity is defined as the amount of enzyme necessary to decrease by 50% the rate of adrenalin autooxidation at pH 10.2. Manganese SOD (MnSOD) activity was determined by incubating the samples with 8 mM KCN. Copper-zinc SOD (CuZnSOD) activity was calculated as the difference between total SOD and MnSOD activities. The activity of catalase (CAT) was determined by the rate of H_2O_2 disappearance measured at 240 nm, according to Claiborne (1985). One unit of CAT activity is defined as the amount of enzyme that decomposes 1 mmol H_2O_2 per minute at 25°C and pH 7.0. The activity of glutathione peroxidase (GSHPx) was determined by the GSH-dependent reduction of t-butyl hydroperoxide, using a modification of the assay described by Paglia and Valentine (1967). One unit of GSHPx activity is defined as the amount needed to oxidize 1 nmol NADPH per min at 25°C and pH 7.0. Glutathione reductase (GR) activity was determined using the method of Glatzle *et al*. (1974). This assay is based on NADPH oxidation concomitant with GSH reduction. One unit of GR activity is defined as the oxidation of 1 nmol NADPH per min at 25°C and pH 7.4. All enzyme activities were expressed as units·mg^{-1} protein.

To evaluate changes in enzyme activities, two types of controls were used. One control (C0h) comprised uteri frozen in liquid nitrogen immediately after dissection. A second control (C2h) comprised uteri frozen in liquid nitrogen after their incubation for an equivalent experimental time (2 h) at 37°C without any addition of H_2O_2 (C2h). Within the latter control, two groups existed: spontaneously active and Ca^{2+}-(6 mM) activated.

Data analysis and statistical procedures

Statistical analyses (descriptive statistics, analysis of variance – ANOVA) were performed according to protocols described by Hinkle *et al*. (1994) and Manley (1986) using Statistical Analysis Software (SAS version 9.1.3, SAS Institute, Cary, NC, USA). The effect of H_2O_2 on uterine contractility was tested by two-way ANOVA (factors: H_2O_2 concentration and type of activation) and regression analysis (the minimum level of significance was when $P < 0.05$). Data were linearly fitted and compared by the *F*-test. The effect of H_2O_2 treatment on anti-oxidative defence enzymes was tested by one-way ANOVA, and the data were compared using the unequal N honestly significant difference *post hoc* test. The action of different inhibitors on the effect of H_2O_2 on uterine contractility was tested by main effect two-way ANOVA (factors: H_2O_2 concentration and the presence of inhibitors). *Post hoc* comparison employed Duncan's range test and regression analysis. Linear fits of data sets were compared by the *F*-test.

Materials

The following were used: H_2O_2 (ZORKA Pharma, Sabac, Serbia); propranolol, MB, L-NAME, TEA, glibenclamide and 4-AP (Sigma Chemical Co., St Louis, MO, USA). All were dissolved in distilled water except for glibenclamide, which was dissolved in polyethylene glycol. De Jalon's solution contained (in g·L^{-1}): NaCl 9.0, KCl 0.42, $NaHCO_3$ 0.5, $CaCl_2$ 0.06 and glucose 0.5.

Results

Effect of H_2O_2 on spontaneous and Ca^{2+}-induced contractions of isolated rat uteri

H_2O_2 (2, 20, 200, 400 μM and 3 mM) caused concentration-dependent relaxation of both spontaneous and Ca^{2+}-induced contractions in isolated rat uteri (Figure 1) (effect of dose, ANOVA, $P < 0.001$ and regression analysis R factor, $P < 0.0001$). There was no significant difference between contraction types.

Changes in anti-oxidative enzyme activity in spontaneously active isolated rat uteri treated with H_2O_2

In spontaneously active rat uteri, higher GSHPx activity was found after H_2O_2 treatment, compared with both rat uteri immediately frozen after dissection (C0h) and untreated spontaneously active rat uteri incubated for an equivalent time (C2h) (Figure 2). CAT activity was decreased in the C2h samples compared with the levels in the C0h samples, but there were no significant changes after H_2O_2 treatment. MnSOD activity was increased after H_2O_2 incubation compared with activity in the C2h samples, but H_2O_2 suppressed the elevation of CuZnSOD activity found in the C2h samples. Note that a significant difference was found in CuZnSOD activities between the C0h and C2h samples. Treatment with H_2O_2 had no effect on GR activities in spontaneously contracting rat uteri (C0h vs. C2h samples).

Changes in anti-oxidative enzyme activity in Ca^{2+}-induced isolated rat uteri treated with H_2O_2

In uteri with Ca^{2+}-induced contractions, H_2O_2 treatment led to lower GR and higher MnSOD and GSHPx activities (Figure 3). As observed with spontaneously active uteri, H_2O_2 suppressed the elevation of CuZnSOD activity found in the C2h samples. Here, in the presence of Ca^{2+}, a significant difference was found in CuZnSOD activities between the C0h and the C2h samples. There were no changes in the activity of CAT in any of the groups of uteri.

Effect of H_2O_2 on spontaneous contractions of isolated rat uteri in the presence of L-NAME, propranolol and MB

H_2O_2 induced dose-dependent relaxation of spontaneously active rat uteri contractions in the presence of L-NAME (10 μM), propranolol (1 μM) and MB (0.4 μM). There was a significant effect of H_2O_2 concentration (ANOVA, $P < 0.001$, Figure 4A and regression analysis R factor $P < 0.001$, Figure 4B). MB increased the relaxation effect of H_2O_2 (ANOVA $P < 0.05$).

Effect of H_2O_2 on Ca^{2+}-induced contractions of isolated rat uteri in the presence of L-NAME, propranolol and MB

H_2O_2 induced dose-dependent relaxation of Ca^{2+}-induced rat uteri contractions in the presence of L-NAME (10 μM),

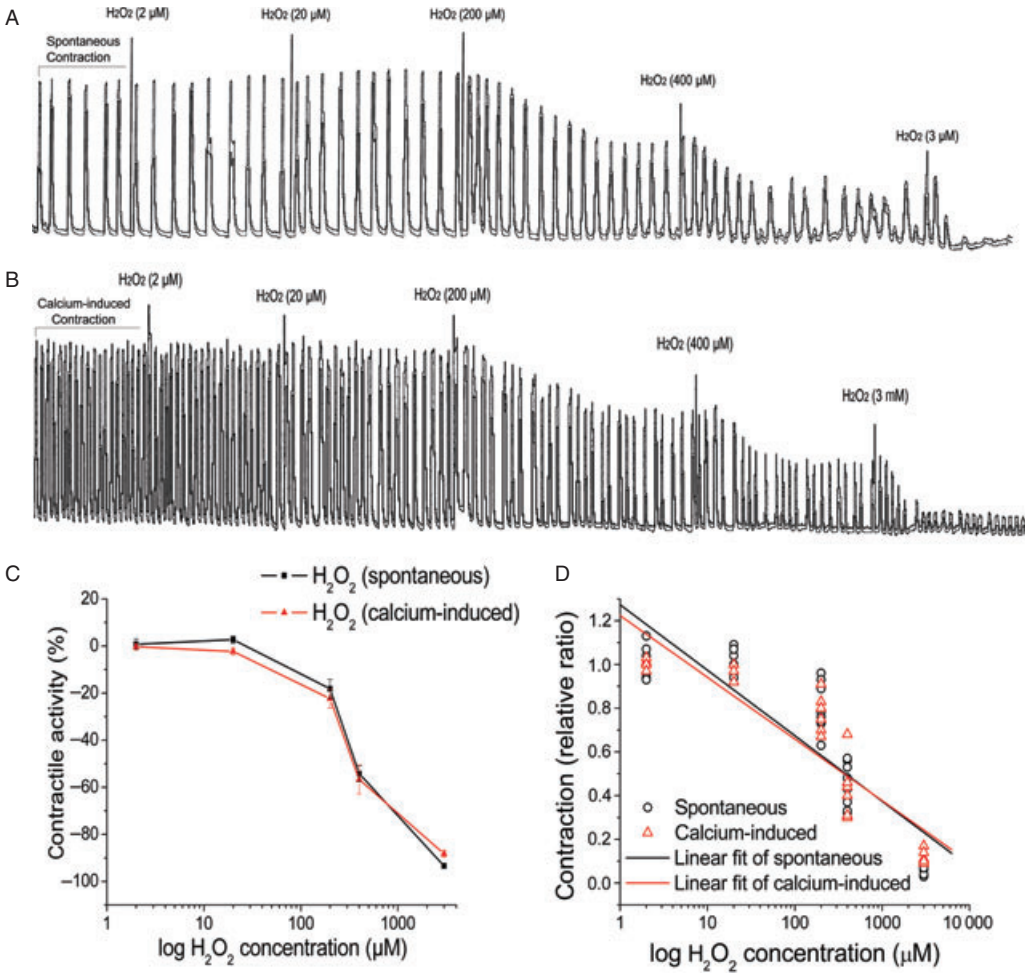

Figure 1 (A) A representative original trace of spontaneous uterine contractions treated with H_2O_2 (2, 20, 200, 400 μM and 3 mM). (B) A representative original trace of Ca^{2+}-induced uterine contractions treated with H_2O_2 (2, 20, 200, 400 μM and 3 mM). (C) Contractile activity of spontaneous and Ca^{2+}-induced rat uteri treated with H_2O_2 (2, 20, 200, 400 μM and 3 mM). Contractile activity was expressed as the relative ratio between mean height peak of untreated control and treated uteri. Data are expressed as mean ± SEM ($n = 8$). Data were analysed by two-way ANOVA (factors: type of contractions and H_2O_2 dose), and showed significant dose effect ($F = 380$, $P < 0.001$) and non-significant type of contractions effect ($F = 0.7$). (D) Linear fit and regression analysis of contractile activity of spontaneous and Ca^{2+}-induced rat uteri treated with H_2O_2 (2, 20, 200, 400 μM and 3 mM). Data are presented as individual points. H_2O_2 dose effect was significant for both types of contractions ($P < 0.0001$). There were no statistical differences between slopes and correlation coefficients of spontaneous ($R = -0.88 \pm 0.18$) and Ca^{2+}-induced ($R = -0.90 \pm 0.16$) linear fitted lines (*F*-test).

propranolol (1 μM) and MB (0.4 μM), with a significant H_2O_2 concentration effect (ANOVA, $P < 0.001$; regression analysis R factor $P < 0.0001$). Also, the presence of MB increased the relaxation effect of H_2O_2 ($P < 0.01$), while L-NAME antagonized the relaxation effect of H_2O_2, but only at lower doses ($P < 0.01$) (Figure 5A,B).

Effect of H_2O_2 on spontaneous contractions of isolated rat uteri in the presence of TEA, glibenclamide and 4-AP

H_2O_2 induced dose-dependent relaxation of spontaneously active rat uteri in the presence of TEA (6 mM), glibenclamide (6 μM) and 4-AP (1 mM). There was a significant effect of H_2O_2 concentration (ANOVA $P < 0.001$; regression analysis R factor $P < 0.0001$). All these antagonists altered H_2O_2-induced relaxation (ANOVA treatment effect $P < 0.001$; regression analyses, significant differences in slopes and intercepts, $P < 0.001$). At low H_2O_2 concentrations, the antagonists potentiated H_2O_2-induced relaxation, whereas at higher concentrations (above 200 μM H_2O_2), the antagonists attenuated H_2O_2-induced relaxations (Figure 6A). The relaxation effect was more profound in the presence of 4-AP than in the presence of TEA (significant difference $P < 0.001$ by Duncan's *post hoc* test, as well as significant differences between linear fit of curves calculated by *F*-tests) (Figure 6B).

Effect of H_2O_2 on Ca^{2+}-induced contractions of isolated rat uteri in the presence of TEA, glibenclamide and 4-AP

The effect of H_2O_2 on Ca^{2+}-induced uterine activity was similar to that found in spontaneously active uteri in the presence of TEA (6 mM), glibenclamide (6 μM) and 4-AP (1 mM) (ANOVA; $P < 0.001$ regression analysis R factor $P < 0.0001$). Also, either TEA or 4-AP changed H_2O_2-induced relaxation (ANOVA treatment effect $P < 0.001$; regression analyses, linear fit, *F*-test significant differences, $P < 0.001$). At a low H_2O_2 concentra-

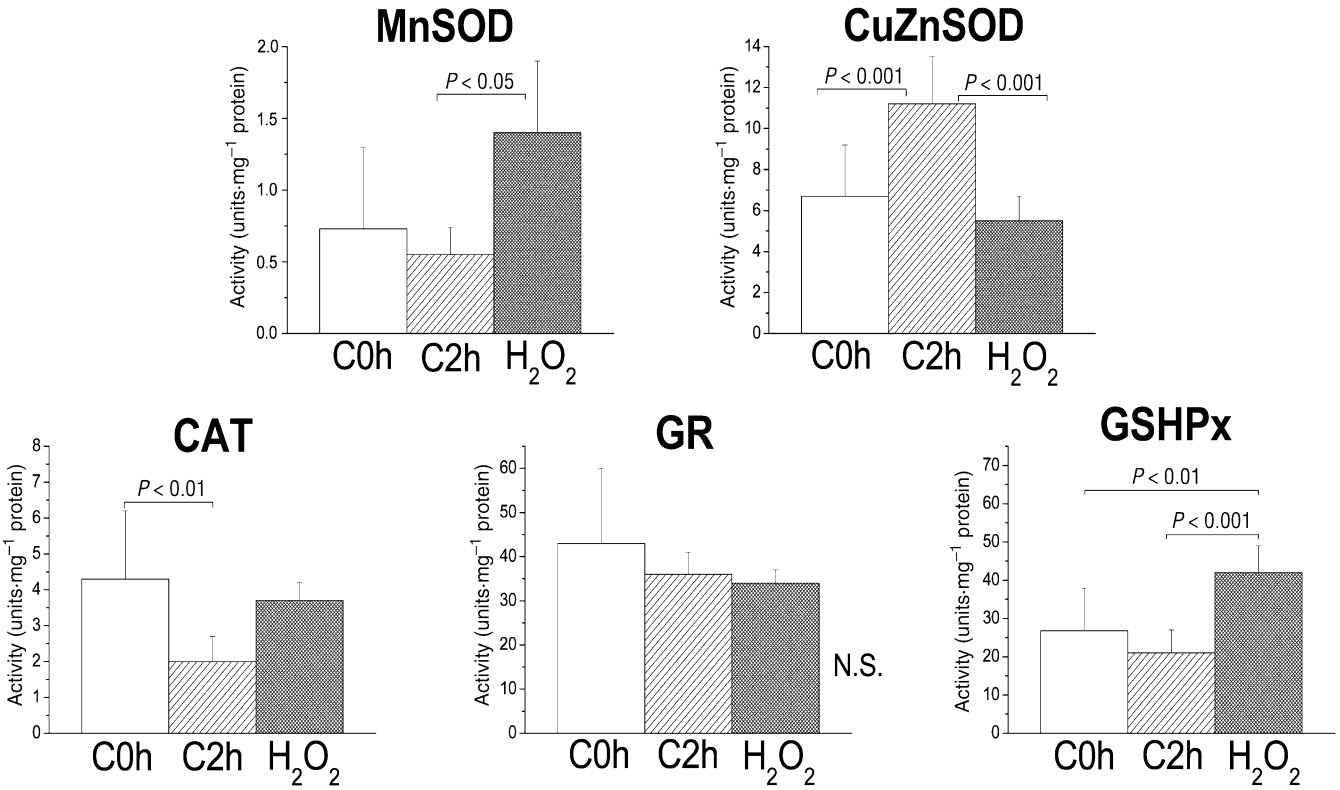

Figure 2 Anti-oxidant enzyme activities in spontaneously active rat uteri. Enzyme activities were determined in untreated rat uteri immediately frozen after dissection (control, C0h, $n = 8$), in untreated ispontaneously active rat uteri incubated for the equivalent experimental time (2 h at 37°C) without the addition of H_2O_2 (C2h, $n = 10$) and spontaneously active rat uteri incubated for 2 h at 37°C treated with increasing concentration of H_2O_2 ($n = 7$). Data are expressed as mean ± SEM. The groups were compared by one-way ANOVA ($P < 0.05$ was considered as significant) followed by honestly significant difference *post hoc* test for unequal n (n-number of samples). Probability levels are presented to denote the individual differences. Concentrations of H_2O_2: 2, 20, 200, 400 μM, 3 mM. CAT, catalase; CuZnSOD, copper-zinc superoxide dismutase; GR, glutathione reductase; GSHPx, glutathione peroxidase; MnSOD, manganese superoxide dismutase.

tion, these antagonists potentiated H_2O_2-induced relaxation, whereas at higher concentrations (above 200 μM H_2O_2), the antagonists attenuated H_2O_2-induced relaxation (Figure 7A). The relaxation effect was more profound in the presence of 4-AP than TEA (significant difference $P < 0.05$ by Duncan's *post hoc* test, as well as significant differences between slopes and intercepts calculated by regression analysis) (Figure 7B). The effects of glibenclamide on H_2O_2-induced relaxation of Ca^{2+}-induced uterine activity were apparent only at H_2O_2 concentrations higher than 200 μM (Figure 7A).

Discussion and conclusions

Our results provide evidence that H_2O_2 caused a dose-dependent decrease in the contractions of rat isolated uteri, independent of the type of activation (spontaneous or Ca^{2+}-induced). An anti-oxidant response to the administered H_2O_2 (independent of the type of activation) was also observed. Furthermore, higher GSHPx activity and suppression of elevated CuZnSOD activity occurred during incubations. A previous study on porcine endothelial aortic cells treated with H_2O_2 indicated increased superoxide anion ($O_2\bullet^-$) (Coyle *et al.*, 2006) and decreased content of GSH (Witting *et al.*, 2006), the latter being a necessary factor for GSHPx action in scavenging H_2O_2. These reports are in agreement with our results, indicating that the changes in the responses of the uteri can be seen as an attempt to defend against disrupted homeostasis and prevent damage by oxidative stress. Changes in GSHPx activity without changes in GR activity increase H_2O_2 elimination from the spontaneously active uteri, leading to changes in the cellular redox state due to an increased GSSG/GSH ratio. A previous study has shown that SOD isoforms may act to preserve endothelium-dependent relaxation not only by increasing the half-life of endothelium-derived relaxing factor (NO) via removal of $O_2\bullet^-$, but also by converting $O_2\bullet^-$ into H_2O_2 that can exhibit EDHF activity (Morikawa *et al.*, 2003). SOD's main activity, dismutation of $O_2\bullet^-$ and production of H_2O_2, is accompanied by activity towards NO and its redox congeners. Taking into account that CuZnSOD can catalyse the reversible conversion of nitroxyl anion (NO^-) to NO (Murphy and Sies, 1991), while MnSOD may catalyse NO dismutation and the generation of peroxynitrite and H_2O_2 (Filipovic *et al.*, 2007), a complex network of possible actions of these enzymes in uterine smooth muscle can be established. This could be the case and in the myometrium, relaxation prevails via conversion of $O_2\bullet^-$ to H_2O_2 by SOD. In fact, we found that after exogenous addition of H_2O_2, CuZnSOD activity was decreased. The initial cause of such effect could be direct inhibition by H_2O_2 (Hodgson and Fridovich, 1975). On

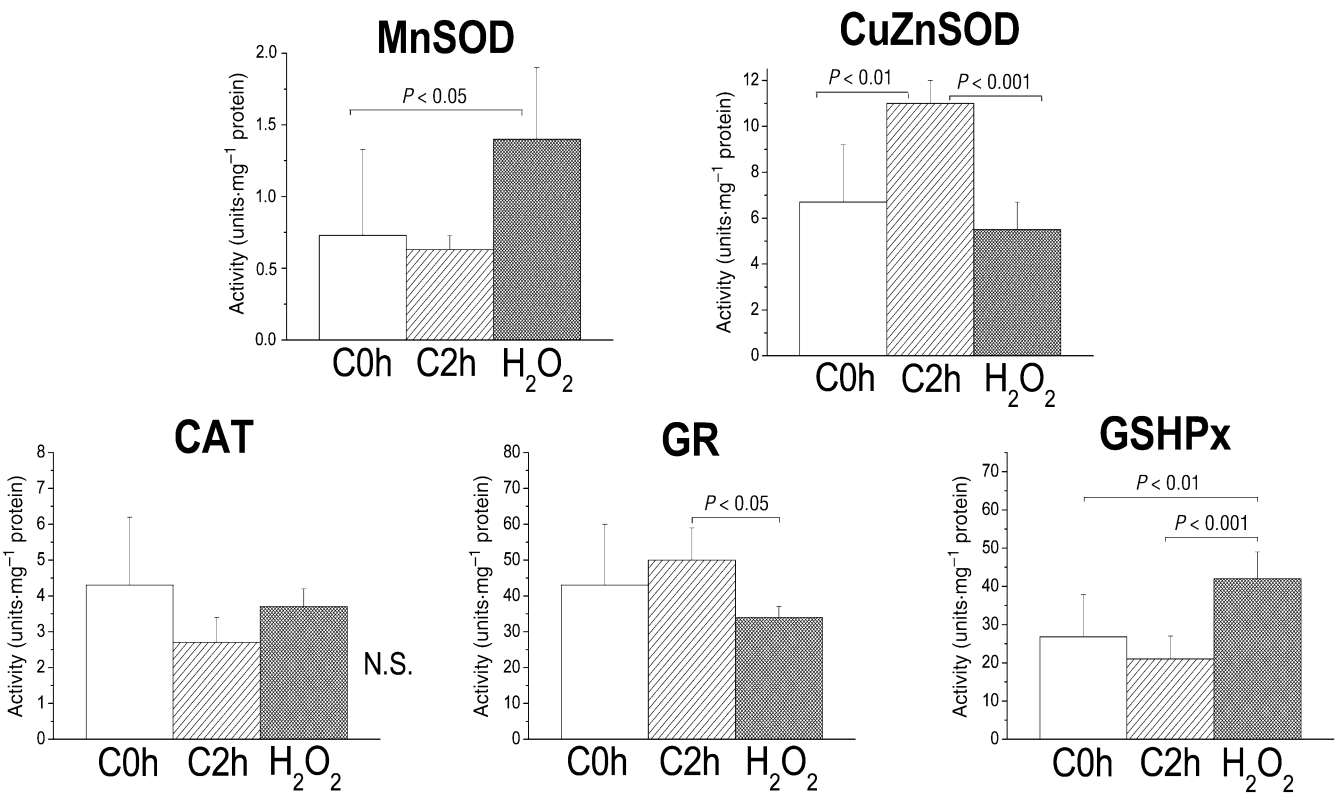

Figure 3 Anti-oxidant enzyme activities in Ca^{2+}-activated rat uteri. Enzyme activities were determined in untreated isolated rat uteri immediately frozen after dissection (control, C0h, $n = 8$), in untreated isolated Ca^{2+}-activated rat uteri incubated for the equivalent experimental time (2 h at 37°C) without the addition of H_2O_2 (C2h, $n = 10$) and isolated Ca^{2+}-activated rat uteri incubated for 2 h at 37°C treated with increasing concentration of H_2O_2, ($n = 7$). Data are expressed as mean ± SEM. The groups were compared by one-way ANOVA ($P < 0.05$ was considered as significant) followed by the honestly significant difference *post hoc* test for unequal *n* (*n*-number of samples). Probability levels are presented to denote the individual differences. Concentrations of H_2O_2: 2, 20, 200, 400 μM and 3 mM. CAT, catalase; CuZnSOD, copper-zinc superoxide dismutase; GR, glutathione reductase; GSHPx, glutathione peroxidase; MnSOD, manganese superoxide dismutase.

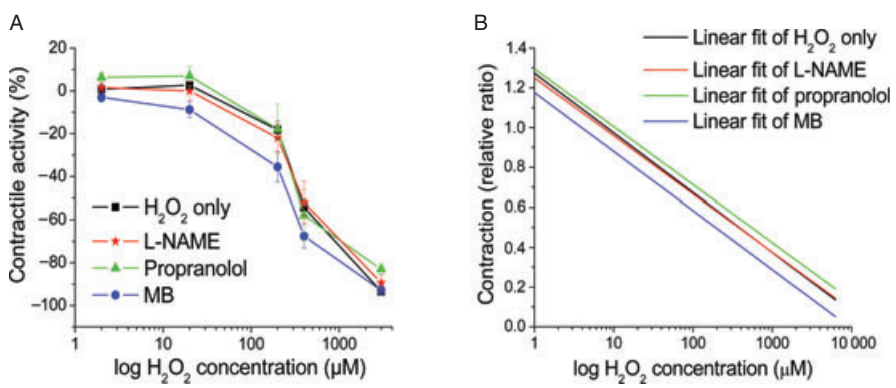

Figure 4 (A) Effect of H_2O_2 (2, 20, 200, 400 μM and 3 mM) on spontaneous contractile activity of rat uteri in the presence of N^{ω}-nitro-L-arginine methyl ester (L-NAME) 10 μM, propranolol 1 μM and methylene blue (MB) 0.4 μM. Contractile activity was expressed as the relative ratio between mean height peak of untreated control and treated uteri. Data are expressed as mean ± SEM ($n = 8$); Results were compared by two-way ANOVA (factors: treatment and H_2O_2 concentration). There were significant H_2O_2 dose ($F = 187.2$, $P < 0.001$) and treatment effects ($F = 2.9$, $P < 0.05$). *Post hoc* comparison showed that MB treatment was significantly different compared with the other treatments ($P < 0.05$). (B) Linear fit of data presented in Figure 4A. Regression analysis of effects of H_2O_2 (2, 20, 200, 400 μM and 3 mM) on spontaneous contractile activity of rat uteri in the presence of L-NAME (10 μM), propranolol (1 μM0 and MB (0.4 μM) showed significant effect of H_2O_2 dose ($P < 0.0001$). There was no difference between correlation coefficients of treatments: H_2O_2 (–0.88 ± 0.18), L-NAME (–0.80 ± 0.25), propranolol (–0.78 ± 0.25) and MB (–0.89 ± 0.17) (mean ± SD).

the other hand, MnSOD activity was increased. Recent studies in SOD2 over-expressing mice have shown that the forced depression of mouse fibres (and rat fibres) was mainly due to decreased myofibrilar Ca^{2+} sensitivity, and was explained by a greater capacity of these fibres to convert $O_2^{\bullet-}$ to H_2O_2 during fatiguing stimulation (Bruton *et al*., 2008).

We found that the β-adrenoceptor signalling pathway was not operative in H_2O_2-induced rat uterine relaxation, as pro-

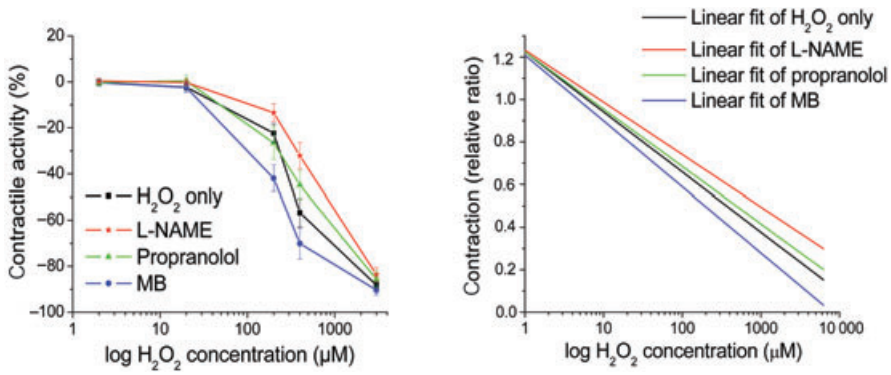

Figure 5 (A) Effect of H_2O_2 (2, 20, 200, 400 μM and 3 mM) on Ca^{2+}-induced contractile activity of rat uteri in the presence of N^{ω}-nitro-L-arginine methyl ester (L-NAME) (10 μM), propranolol (1 μM0) and MB (0.4 μM). Contractile activity was expressed as the relative ratio between mean height peak of untreated control and treated uteri. Data are expressed as mean ± SEM (n = 8); Results were compared by two-way ANOVA (factors: treatment and H_2O_2 concentration). There were significant H_2O_2 dose (F = 278.2, P < 0.001) and treatment effects (F = 10.4, P < 0.001). *Post hoc* comparison showed that MB treatment was significantly different compared with H_2O_2 treatment (P < 0.001). (B) Linear fit of data presented in Figure 5A. Regression analysis of effects of H_2O_2 (2, 20, 200, 400 μM and 3 mM) on Ca^{2+}-induced contractile activity of rat uteri in the presence of L-NAME (10 μM), propranolol (1 μM) and MB (0.4 μM) showed significant effect of H_2O_2 dose (P < 0.0001). There were no differences between correlation coefficients of treatments: H_2O_2 (–0.90 ± 0.16), L-NAME (–0.78 ± 0.22), propranolol (–0.87 ± 0.17) and MB (–0.90 ± 0.17) (mean ± SD). *F*-test showed that at the 0.05 significance level, the fitted lines were not statistically different.

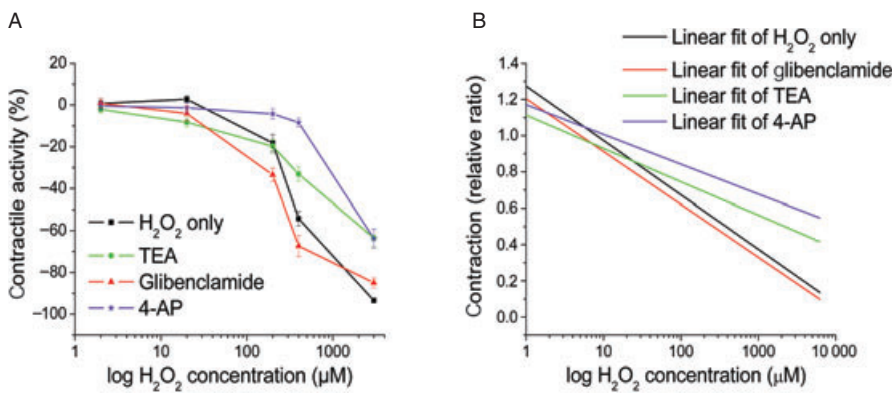

Figure 6 (A) Effect of H_2O_2 (2, 20, 200, 400 μM and 3 mM) on spontaneous contractile activity of rat uteri in the presence of tetraethylamonium (TEA) (6 mM), glibenclamide (6 μM) and 4-aminopyridine (4-AP) (1 mM). Contractile activity was expressed as the relative ratio between mean height peak of untreated control and treated uteri. Data are expressed as mean ± SEM (n = 8); results were compared by two-way ANOVA (factors: treatment and H_2O_2 concentration). There were significant H_2O_2 dose (F = 207, P < 0.001) and treatment effects (F = 17.1, P < 0.001). *Post hoc* comparison showed that all the treatments significantly altered H_2O_2 response (P < 0.001). (B) Linear fit of data presented in Figure 6A. Regression analysis of effect of H_2O_2 (2, 20, 200, 400 μM and 3 mM) on spontaneous contractile activity of rat uteri in the presence of TEA (6 mM), glibenclamide (6 μM) and 4-AP (1 mM) showed a significant effect of H_2O_2 dose (P < 0.0001). Correlation coefficient of 4-AP treatment (–0.72 ± 0.18) was different from the others: H_2O_2 (–0.88 ± 0.18), glibenclamide (–0.91 ± 0.15) and TEA (–0.87 ± 0.12) (mean ± SD). *F*-test analysis revealed that linear fit of 4-AP and TEA data were statistically significant compared with H_2O_2 (both P < 0.001).

pranolol did not antagonize the effect of H_2O_2 on either type of activation. In addition, we cannot rule out possible modifications of associated signalling regulators (such as K_v channels) by tyrosine kinases (Nitabach *et al.*, 2002) and tyrosine phosphatases (Mason *et al.*, 2002).

L-NAME partially inhibited H_2O_2-induced relaxation in Ca^{2+}-induced rat uteri. The ability of H_2O_2 to up-regulate endothelial NO synthase expression has already been demonstrated in arteries (Thomas *et al.*, 2002), and in part explains previous findings that the expression levels of endothelial NO synthase protein are paradoxically increased during exercise training in mice (Laursen *et al.*, 2001). MB did not inhibit H_2O_2-induced relaxation. In contrast, it potentiated the effect of H_2O_2 on both types of activation. This observed potentiating effect may be explained by the fact that MB, apart from being an inhibitor of the cGMP signalling pathway, is a redox-cycling agent that produces H_2O_2 at the expense of O_2 and NAD(P)H in each cycle (Buchholz *et al.*, 2008).

Support for a possible relaxation mechanism, independent of cGMP signalling, for H_2O_2 can be found in a study by Burgoyne *et al.* (2007). An alternative mechanism, which could induce vasorelaxation in parallel to the classical activation involving NO and cGMP, would entail H_2O_2 operating as an EDHF by directly activating PKG (cGMP-dependent protein kinase), resulting in phosphorylation and activation of K^+ channels. Recent studies have suggested that the nitroxyl anion (NO^-) can activate K_v channels independently of cGMP (Costa *et al.*, 2001; Irvine *et al.*, 2003), and it is

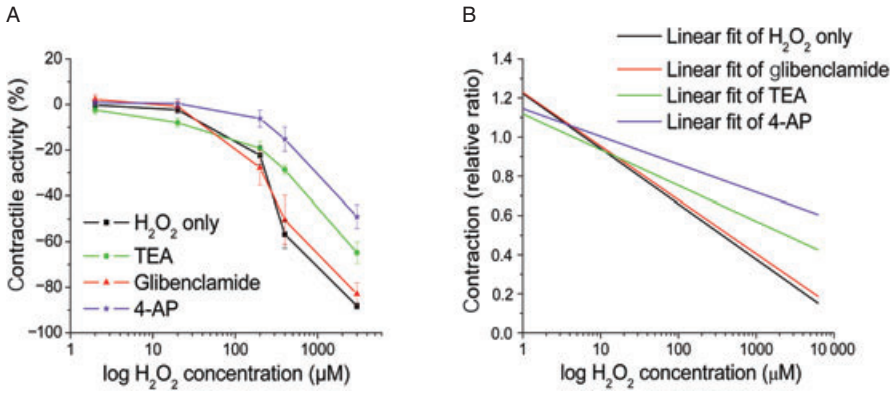

Figure 7 (A) Effect of H_2O_2 (2, 20, 200, 400 μM and 3 mM) on Ca^{2+}-induced contractile activity of rat uteri in the presence of tetraethylamonium (TEA) (6 mM), glibenclamide (6 μM) and 4-aminopyridine (4-AP) (1 mM). Contractile activity was expressed as the relative ratio between mean height peak of untreated control and treated uteri. Data are expressed as mean ± SEM (n = 8); There were significant H_2O_2 dose (F = 146.2, P < 0.001) and treatment effects (F = 16.1, P < 0.001). *Post hoc* comparison showed that TEA and 4-AP pretreatments were significantly different compared with H_2O_2 only treatment (P < 0.001). Glibenclamide treatment had effect, but at the concentrations above 200 μM (P < 0.01). (B) Linear fit of data presented in Figure 7A. Regression analysis of effect of H_2O_2 (2, 20, 200, 400 μM and 3 mM) on Ca^{2+}-induced contractile activity of rat uteri in the presence of TEA (6 mM), glibenclamide (6 μM), 4-AP (1 mM) showed significant effect of H_2O_2 dose (P < 0.0001). Correlation coefficient of 4-AP treatment (–0.71 ± 0.16) was different from the others: H_2O_2 (–0.90 ± 0.16), glibenclamide (–0.83 ± 0.21) and TEA (–0.86 ± 0.12) (mean ± SD). *F*-test analysis revealed that the linear fit of 4-AP data was statistically significant compared with H_2O_2 (P < 0.05).

well-known that the vasodilator response, previously attributed to NO, is in part mediated by NO^- (Ellis *et al.*, 2000; Wanstall *et al.*, 2001). In the study by Irvine *et al.* (2003), the inhibitory effect of 4-AP on NO^--mediated relaxation is indicative of the activation of K_v channels by NO^- and subsequent smooth muscle hyperpolarization. In addition, their findings concur with the study in isolated sheep urethra, where relaxation responses to NO^- were impaired in part by 4-AP (Costa *et al.*, 2001). Therefore, our observed increase in MnSOD activity in rat uteri treated with H_2O_2, together with the fact that MnSOD-catalyses NO dismutation into NO species [nitrosonium (NO^+) and NO^- (Filipovic *et al.*, 2007)], may indicate that H_2O_2-induced relaxation in uteri may require this indirect mechanism over that involving NO^-. Further studies are required to fully understand such speculation.

To examine if K^+ channels were involved in H_2O_2-induced relaxation of rat uteri, we performed a variety of experiments using K^+ channel blockers. Our results showed that all the used antagonists had effects, but with the potency order 4-AP > TEA > glibenclamide (the latter far less effective). These results indicate that H_2O_2-mediated uterine relaxation involved K^+ channels. In the presence of K^+ channel antagonists, higher doses of H_2O_2 are required to reduce uterine contractions compared with L-NAME, MB and propranolol, suggesting that H_2O_2-mediated relaxation of uterine smooth muscle is mediated predominantly through K^+ channels. As glibenclamide reduced the relaxation effect of H_2O_2 to a lower extent than 4-AP (which significantly inhibited it), we can conclude that K_v channels most likely play the most significant role in H_2O_2-induced smooth muscle relaxation. These results are similar to those obtained by other investigators that employed arterial smooth muscles treated with H_2O_2 (Rogers *et al.*, 2006).

An important aspect underlying signalling properties of H_2O_2 is its ability to target proteins containing oxidation-susceptible cysteine residues critical for protein function. Oxidized thiol groups can interact with nearby cysteine residues to form a disulphide bridge. Several electrophysiological studies have suggested that H_2O_2 targets a cysteine residue in the modulatory subunit of K_v channels and have identified a specific cysteine residue that confers redox-sensitivity to the channel (Rettig *et al.*, 1994; Wang *et al.*, 1996). Other studies have also demonstrated that H_2O_2 oxidizes a vascular thiol target activating K_v-channels, leading to subsequent relaxation (Rogers *et al.*, 2006). Therefore, H_2O_2 may directly interact with K_v channels in uteri.

In conclusion, our results demonstrate that exogenous H_2O_2 causes relaxation of rat uteri independent of the type of activation (spontaneous or Ca^{2+}-induced). Voltage-dependent K^+ channels may represent the main target for H_2O_2-induced relaxation in rat uteri. K_v channel activation by H_2O_2 may involve an indirect mechanism via reactive nitrogen species or via direct activation by H_2O_2 targeting protein thiol groups within K_v-channels (or proteins that regulate them). Further studies are required to reveal which components are specifically affected by H_2O_2. Increased endogenous GSHPx activity indicated that the uteri opposed a state of disrupted homeostasis and attempted to prevent damage via oxidative stress.

Acknowledgements

This work was supported by a grant from the Ministry of Science of the Republic of Serbia, project no: 143034B 'The Role of Redox-Active Substances in the Maintenance of Homeostasis'. Manuscript comments and suggestions from Professor Vladislav Varagic were much appreciated.

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