Research Article

Reversible Oxidation of Myometrial Voltage-Gated Potassium Channels with Hydrogen Peroxide

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The uteri, spontaneously active or Ca2+ (6 mM) induced, were allowed to equilibrate, and to inhibit voltage-gated potassium (KV) channels 1 mM 4-amino pyridine (4-AP) was applied for 15 min before adding H2O2. H2O2 was added cumulatively: 2 μM, 20 μM, 200 μM, 400 μM, and 3 mM. Average time for H2O2 concentrations (2, 20, 200, and 400) μM to reach its full effect was 15 min. H2O2 3 mM had a prolonged effect and therefore was left to act for 30 min. Two-way ANOVA showed significant differences in time dependency between spontaneous and Ca2+-induced rat uteri after applying 3 mM H2O2 (type of contraction, \( P = 0.0280 \)), but not 400 μM H2O2 (\( P = 0.9271 \)). Our results indicate that H2O2 oxidises channel intracellular thiol groups and activates the channel, inducing relaxation. Cell antioxidative defence system quickly activates glutathione peroxidase (GSHPx) defence mechanism but not catalase (CAT) defence mechanism. Intracellular redox mechanisms repair the oxidised sites and again establish deactivation of KV channels, recuperating contractility. In conclusion, our results demonstrate that KV channels can be altered in a time-dependent manner by reversible redox-dependent intracellular alterations.

1. Introduction

Several studies have reported that hydrogen peroxide (H2O2) can mediate smooth muscle relaxation as an endothelium-derived hyperpolarising factor (EDHF) via activation of potassium (K+) channels [1–4]. Activation of K+ channels leads to hyperpolarisation and lowering of the calcium (Ca2+) concentration, resulting in a smooth muscle relaxation. To date, several subtypes of K+ channels have been identified in the rat uteri smooth muscle. The most abundant and most well studied include large conductance Ca2+- and voltage-sensitive K+ channels (BKCa), ATP-dependent K+ channels (KATP), voltage-dependent K+ channels (KV), and small-conductance Ca2+-sensitive K+ channels (SK). Our previous study showed that H2O2 induces relaxation in the smooth muscle of rat uteri [5]. In an attempt to identify the signaling pathways used by H2O2 in this tissue, we then performed a variety of experiments using a range of inhibitors: Nω-nitro-L-arginine methyl ester (L-NAME; NOS inhibitor), methylene blue (MB; cGMP signalling pathway inhibitor), propranolol (non-selective β-adrenoceptor antagonist), tetraethylammonium (TEA; nonselective K+ channel inhibitor), glibenclamide (selective ATP dependent K+ channel inhibitor), and 4-aminopyridine (4-AP; voltage-dependent K+ channel inhibitor). Our results indicated that H2O2-induced uterine relaxation is mediated predominantly through K+ channels as in the presence of K+ channel antagonists, higher doses of H2O2 were required to reduce uterine contractions compared with L-NAME, MB, and propranolol. The potency order of the K+ channels inhibitor effect was 4-AP > TEA > glibenclamide (the latter being far less effective), indicating that KV channels play the most significant role of K+ channels in H2O2-induced smooth muscle relaxation of rat uteri [5]. These results were similar to those obtained by other investigators that employed arterial smooth muscles treated with H2O2 [6]. KV channels are the biggest family
of potassium channels. They include about 40 members divided in 12 subfamilies, Kv1–Kv12, of which Kv5, Kv6, Kv8, and Kv9 are not independently functional, but are Kv2 channels modulators. Smith and coworkers showed expression of many Kvα subunits in nonpregnant and pregnant mouse myometrium [7]. Opening of Kv channels liberates positive charge leading to membrane repolarisation [8] and relaxation. Response to Kv channel inhibitor 4-AP disappeared in pregnant myometrium, what was correlated to loss of Kv4.3α expression [9], what is probably oestrogen dependent [7].

H2O2 is uncharged oxidant that can diffuse easily through cell membranes being an eligible signal molecule in many physiological responses. Role of H2O2 in the regulation of myometrium smooth muscle contractile activity is not fully resolved and is still under investigation. In intact single fibers, there is evidence of complex multifactorial effects in response to H2O2. For instance, myofibrillar Ca2+ sensitivity increases early during exposure to high H2O2 concentrations and then declines. Moreover, H2O2 has little immediate effect on intracellular Ca2+, but prolonged exposure to H2O2 leads to decreased sarcoplasmic reticulum (SR) Ca2+ reuptake and increased resting (Ca2+), suggestive of loss of Ca2+ homeostasis [10], also the opening probability of SR Ca2+ release channels increases after thiol oxidation [11]. (Ca2+)i increase is a constant feature of pathological states associated with oxidative stress [12]. Recent studies have underscored the notion that the Ca2+ and ROS signalling systems are intimately integrated such that Ca2+-dependent regulation of components of ROS homeostasis might influence intracellular redox balance and vice versa [13].

In this study, we have further examined the mechanism of Kv channels-dependant H2O2-relaxing effect on rat smooth muscle contractile activity and correlated these effects with changes in endogenous antioxidative defence, with respect to two types of activation: spontaneous and calcium-induced.

2. Material and Methods

2.1. Experimental System. Isolated uteri from virgin Wistar rats (200–250 g) in estrous, determined by examination of a daily vaginal lavage [14], were used. All protocols for handling rats were approved by the local ethics committee for animal experimentation that strictly follows international regulations.

2.2. Isolated Organ Bath Study of Uterine Kv Channels Time-Dependent Inhibition. 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 aerated with 95% oxygen and 5% carbon dioxide at 37°C.

The uteri, spontaneously active or Ca2+ (6 mM)-induced, were allowed to equilibrate at 1 g tension before addition of the experimental drugs. To inhibit Kv channels in uteri 1 mM 4-amino pyridine (4-AP) was applied for 15 min before adding H2O2. H2O2 was added cumulatively: 2 μM, 20 μM, 200 μM, 400 μM, and 3 mM. Myometrial tension was recorded isometrically with a TSZ-04-E isolated organ bath and transducer (Experimetria, Budapest, Hungary). Each H2O2 concentration was left to act for 15 min except 3 mM that was left for 30 min.

2.3. Determination of Uterine Antioxidative Enzyme Activities after High Impact of H2O2. To 7 h contracting uteri were applied 3.6 mM H2O2 (summed cumulative concentrations from previous experiment). Control group were uteri active equivalent time but untreated. After experiment, samples were immediately frozen, using liquid nitrogen, and then transferred to −80°C until enzyme analysis.

Thawed uteri were homogenised and sonicated in 0.25 M sucrose, 1 mM EDTA, 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 adrenalin method [15]. 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 H2O2 disappearance measured at 240 nm, according to Claiborne [16]. One unit of CAT activity is defined as the amount of enzyme that decomposes 1 nmol H2O2 per minute at 25°C and pH 7.0. The activity of glutathione peroxidase (GSHPx) was determined by the GSH-dependent reduction of t-butyli hydroperoxide, using a modification of the assay described by Paglia and Valentine [17]. 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. [18]. 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.

2.4. Statistical Analyses. Results were analysed with statistical GraphPad Prism (version 5.03), GraphPad Software, San Diego, CA, USA. Statistical significance was determined with t-test, one-way ANOVA, and post hoc tests: Dunnett—comparison with a control group, Tukey—comparison among all groups and test for linearity trend; two-way ANOVA and post hoc Bonferroni test; regression analysis: Linear regression and nonlinear regression (dose-dependant inhibition model with variable Hill slope).

2.5. Reagents. The following reagents were used: H2O2 (ZORKA Pharma, Sabac, Serbia); 4-AP (Sigma Chemical Co, St Louis, MO, USA). All were dissolved in distilled water. De Jalon’s solution was comprised of (in gl-1): NaCl 9.0, KCl 0.42, NaHCO3 0.5, CaCl2 0.06, and glucose 0.5.
3. Results

3.1. \( K_V \) Channels Dependent \( H_2O_2 \) Effect on Contractile Activity. Inhibition of \( K_V \) channels with 1 mM 4-amino pyridine (4-AP) increased the basal tonus of uteri contractions (results not shown), confirming the presence of \( K_V \) channels in the uteri and their role in contractions. After rat uterine \( K_V \) channels were blocked with 1 mM 4-AP, \( H_2O_2 \) (400 \( \mu M \) and 3 mM) induced effect on contractile activity, both spontaneous and calcium induced. \( H_2O_2 \) first induced relaxation, after which contractions were gradually recovering. Additionally, \( H_2O_2 \) 3 mM had a prolonged effect and therefore was left to act for 30 min. Average time for other \( H_2O_2 \) concentrations (2, 20, 200, and 400 \( \mu M \)) to reach its full effect was 15 min (Figure 1).

3.2. Time-Dependent Changes in \( K_V \) Channel Inhibition. One-way ANOVA time-dependence analysis of \( H_2O_2 \) (400 \( \mu M \) and 3 mM) induced effect in spontaneous rat uteri showed time significance (time resp.: \( P = 0.0246, P < 0.0001 \)). Post hoc test for linear trend showed significant trend of linear regression (400 \( \mu M \) \( H_2O_2 \): \( r^2 = 0.3031, P = 0.0102 \); 3 mM: \( r^2 = 0.8142, P < 0.0001 \)). In \( Ca^{2+} \)-induced rat uteri, were also shown significant time dependency and linear trend in the effect of 3.6 mM (time: \( P < 0.0001 \); \( r^2 = 0.5800, P < 0.0001 \)) but not with 400 \( \mu M \) (time: \( P = 0.6628 \); linear trend: \( r^2 = 0.2673, P = 0.3712 \)) (Figure 2).

Two-way ANOVA showed significant differences in time dependency between spontaneous and \( Ca^{2+} \)-induced rat uteri after applying 3 mM \( H_2O_2 \) (type of contraction, \( P = 0.0280 \)), but not 400 \( \mu M \) \( H_2O_2 \) (\( P = 0.9271 \)). Regression analysis of fitted lines also showed similar time dependency but different contraction intensity after applying 3 mM \( H_2O_2 \) (similar slope, but different intercepts) between spontaneous and \( Ca^{2+} \)-induced rat uteri, as well as no significant differences after applying 400 \( \mu M \) \( H_2O_2 \) (no differences in slope and intercepts between spontaneous and \( Ca^{2+} \)-induced).

3.3. Changes in Antioxidative Enzyme Activity in Rat Uteri after Impact of \( H_2O_2 \) High Concentration. \( t \)-test analysis for changes of antioxidative enzyme activity after applying 3.6 mM \( H_2O_2 \) (concentration that equals summed cumulative concentrations from previous experiment) comparing to \( Ca^{2+} \)-induced uteri active equal time but without \( H_2O_2 \) treatment showed statistically significant increase of CuZnSOD (\( P = 0.0381 \)) and GSHPx (\( P = 0.0344 \)) activity after impact of 3.6 mM (Figure 3).

4. Discussion

Earlier studies have shown that \( H_2O_2 \) can act as contractile and relaxing agent, tissue dependent [19–21], and in some cases, it exhibits a biphasic effect [2, 22]. Role of \( H_2O_2 \) in the regulation of myometrial contractile activity is not fully resolved and is still under investigation. In some states as in the thrombosis postpartum [23] or during powerful myometrial contractions that restrict blood flow to the uterus, reperfusion/ischemia injury can occur [21, 24] possibly producing high impact of \( H_2O_2 \) on the uteri. In our study, we have observed that in \( K_V \) channel inhibitor (4-AP) pretreated uteri, high concentrations of \( H_2O_2 \) caused relaxation and recovery independent of the type of contractile activity (spontaneous or calcium induced), just with differences in intensity. Characteristic of this effect was its significant linear time dependency. After the first relaxation effect, there was a time dependent recovery of contractile activity. Additionally at 3 mM \( H_2O_2 \), effect lasted longer (30 min) then the equilibration time for other \( H_2O_2 \) concentrations (15 min). It is known that proteins are \( H_2O_2 \) targets and that \( K^+ \) channels and proteins that regulate them are redox-sensitive elements [4]. Main protein modifications include direct oxidation, above all amino acids with thiol groups, as cysteine oxidative glication and carboxylation [25–28]. Several electrophysiological studies showed that \( H_2O_2 \) acts on cysteine of \( K_V \) modulatory subunits and found specific cysteins that determine the channel sensitivity. Mutation of these cysteins impeded \( H_2O_2 \)-dependent channel activation [29]. After oxidation thiol groups may interact with nearby cysteins forming disulfide bonds [30]. Rogers and coworkers [4] showed that \( K_V \) channels are regulated in a redox-sensitive manner as \( H_2O_2 \) 1–10 mM-induced \( K_V \) currents in coronary artery smooth muscle cells were being antagonized by DTT, a thiol reductant, and blocked by NEM, a thiol-alkylating agent; but they did not observe rapid reversibility (i.e., the effect of \( H_2O_2 \) to increase \( K_V \) current was sustained). As a plausible explanation for their result, they suggest that \( H_2O_2 \) may oxidize an extracellular target, rather than a cytoplasmic one that could be repaired by endogenous intracellular reductants. They based this on the assumption that: (1) \( H_2O_2 \) crosses membrane easily and has access to extracellular and intracellular thiol groups; (2) thiol groups of extracellular components are always present outside the cell (e.g., extracellular loops of \( K_V \) channels); (3) intracellular targets include \( K_V \) channels or proteins that regulate them and always remain inside of the cell; (4) intracellular reductants do not cross the membrane but remain inside the cell having access only to intracellular components. Thus, if an extracellular target was oxidized, no “repair” (i.e., reduction) would be possible. Conversely, if an intracellular target was oxidized, it might be repaired by intracellular reducing mechanisms [4]. We believe that this oxidation could take place intracellular as well, since we did observe time dependent recovery of contractile activity after \( H_2O_2 \)-induced relaxation in the presence of 4-AP. 4-AP blocks \( K_V \) channels intracellular [31, 32] and has small association and dissociation time (100 ms). 4-AP binding on the intracellular side of the \( K_V \) channel may be temporarily altered due to high concentration of highly diffusible \( H_2O_2 \). It is possible that \( H_2O_2 \) oxidises channel intracellular thiol groups and activates the channel, inducing relaxation. However, 4-AP gradually reassociates to intracellular channel sites as intracellular redox mechanisms repair the binding site and again establishes deactivation of \( K_V \) channels, recuperating contractility. That way \( H_2O_2 \) could directly interact with \( K_V \) channels on the intracellular side, but this bond could be overcome with time and 4-AP bond reestablished, making the \( H_2O_2 \) effect transient. Aikawa et al. also observed the transient response to high
H$_2$O$_2$ concentrations in time. They showed that after 1 h of exposure to 6 different concentrations of H$_2$O$_2$ (0%, 0.0625%, 0.125%, 0.25%, 0.5%, and 1%), the contractile response of rat bladder smooth muscle decreased progressively to increase in H$_2$O$_2$ concentration [33]. In more recent study, Han at al. also found that increasing the duration of treatment with $3 \times 10^{-4}$ g% H$_2$O$_2$ progressively decreased the contractile responses of the smooth muscle of bladder [34].

With an increase in H$_2$O$_2$, its toxic effect starts to appear. Therefore, timely elimination of messengers is important in cell signalisation. In our study after applying high concentration of H$_2$O$_2$ (3.6 mM) on a long-term activity, we observed increase in CuZnSOD and GSHPx activity. H$_2$O$_2$ is mainly scavenged by CAT and GSHPx. CuZnSOD, is a cytoplasmic O$_2$$^•$- scavenger, and as such implies O$_2$$^•$- increased production. Some researchers also showed that H$_2$O$_2$ causes increase in O$_2$$^•$- [35]. Others have observed decrease of reduced form of glutathione, a necessary factor in GSHPx activity [36]. As mentioned previously, we have observed increased activity of GSHPx, H$_2$O$_2$ scavenger. However, other important H$_2$O$_2$ scavenger, CAT, did not show any changes. It seems that cell antioxidative defence system quickly activates GSHPx defence mechanism but not CAT defence mechanism. CAT is mainly active in peroxisome though some of its activity is also present in mitochondria and endoplasmatic reticulum. CAT, comparing to GSHPx, has lesser affinity for H$_2$O$_2$ and as such is not effective in scavenging low concentrations of H$_2$O$_2$ [37, 38]. Therefore, GSHPx is considered as a H$_2$O$_2$ low concentration scavenger. However, it was shown that CAT is also active at H$_2$O$_2$ lower
Figure 3: Change of AOS enzyme activity (MnSOD (a), CuZnSOD (b), CAT (c), GR (d), and GSHPx (e)) in Ca²⁺-induced rat uteri after applying 3.6 mM H₂O₂. Control group were uteri active equivalent time interval without H₂O₂ treatment. Data are expressed as mean ± error. Groups were compared with t-test (P < 0.05, significant). Ns: non significant; *P < 0.05.
concentrations and that it loses its activity at H$_2$O$_2$ higher concentrations [39, 40], what is implying to a possible cause of the absence in its activity in our study.

In conclusion, our results demonstrate that $K_V$ channels can be altered in a time-dependent manner by possible time and redox-dependent alterations of $K_V$ channels intracellular binding sites or proteins that regulate them and that GSHPx mechanism is the primary scavenging mechanism in this H$_2$O$_2$ conditions. Further studies may help to find the possible solutions in protecting myometrium in pathological states including strong redox impact on the cell, as reperfusion ischemia or thrombosis postpartum.

References


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