Hydrogen peroxide and superoxide anion modulate pregnant human myometrial contractility

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Abstract

Reactive oxygen species (ROS) have the propensity to cause macromolecular damage with consequent modification of cellular function. We investigated the effects of two particular oxidants, superoxide (O_2^-) anions and hydrogen peroxide (H_2O_2), on oxytocin-induced myometrial contractility using biopsies from women undergoing Caesarean section at term gestation. Iso-metric tension recordings were performed and concentration–response curves derived after addition of test agents. A maximal reduction in myometrial contractility to $27.2 \pm 4.5\%$ of control was observed followed application of H_2O_2 . The enzyme scavenger catalase (CAT) reduced the inhibitory effect of H_2O_2 but had little effect at 10-fold lower concentrations. Addition of dialysed xanthine oxidase $\pm$ hypoxanthine significantly inhibited contractility to $23.8.0 \pm 4.2\%$ compared with control. Pre-incubation with superoxide dismutase and CAT diminished this effect. The non-specific potassium channel blocker, tetraethylammonium chloride (1 mM), had no effect on myometrial contractility. We conclude that human myometrium is susceptible to the effects of ROS, which may be produced by reperfusion–ischaemic episodes during labour. Our findings could, in part, explain the weak or prolonged depression of contractions characteristic of myometrial dysfunction culminating in difficult labours.

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Introduction

Untimely birth due to the premature onset of labour may be associated with an increased risk of perinatal death and long-term handicap (Goldenberg 2002, Slattery & Morrison 2002). At the other extreme, poor progress in labour is a key factor for delivery by emergency Caesarean section. Parturition and infection-induced preterm labour resemble inflammatory processes (Gibbs *et al.* 1992, Hunt 1994, Thomson *et al.* 1999) that are linked to the production of reactive oxygen species (ROS) including superoxide (O_2^-), hydrogen peroxide (H_2O_2) and peroxynitrite. ROS may also form during intermittent ischaemia induced by powerful myometrial contractions restricting blood flow to the uterus causing a reperfusion/ischaemia injury (Nakai *et al.* 2000). Zyrianov *et al.* (2003) have also shown increased free radical generation in human myometrium during labour. Moreover, raised serum and placental levels of lipid hydroperoxides have been demonstrated during the progression of normal pregnancy (Little & Gladen 1999).

The response of the uterus to hypoxia is well-documented (Wray 1993, Taggart & Wray 1995). Metabolic inhibition, using cyanide to mimic hypoxia, causes a

reduction in the force of uterine contractions which is accompanied both *in vivo* (Larcombe-McDouall *et al.* 1998) and *in vitro* by intracellular acidification, decreased levels of ATP, phosphocreatine, intracellular Ca^{2+} ($[Ca^{2+}]_i$) and increased inorganic phosphate levels (Wray 1990, Taggart & Wray 1998). These findings have implications for dysfunctional labours and may be associated with the increasing numbers of ‘failure of labour to progress’ cases of which at least one in five births occurs by Caesarean section. In vascular, intestinal and airway smooth muscle, ROS have been found to affect contraction (Bauer *et al.* 1999, Callahan *et al.* 2001, Kimura *et al.* 2002) and contribute to muscle fatigue in skeletal muscle, although the mechanisms involved are incompletely understood. It has also been demonstrated that O_2^- anion production increases $[Ca^{2+}]_i$ in human myometrium, an effect that was abolished in the presence of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) (Masumoto *et al.* 1990).

Large conductance calcium-activated potassium channels (BK_{Ca}) are ubiquitously distributed in smooth muscle, and have been demonstrated to play an important role in regulation of myometrial contractility (Tritart *et al.* 1991,

Anwer *et al.* 1993, Khan *et al.* 1993) by a negative feedback mechanism to limit depolarization and contraction. Activation of BK_{Ca} channels leads to membrane hyperpolarization, which closes voltage-dependent Ca²⁺ channels and reduces Ca²⁺ influx, resulting in a reduction in [Ca²⁺]_i and hence relaxation.

There is increasing evidence that many cellular effects of ROS are mediated by changes in ionic conductance (Kourie 1998). DiChiara & Reinhart (1997) demonstrated that oxidation with H₂O₂ leads to decreased BK_{Ca} channel activity in HEK 293 cells transfected with the brain *hsl* α -subunit which encodes the BK_{Ca} channel. Conversely, H₂O₂ increases BK_{Ca} channel activity to cause relaxation of porcine coronary artery (Barlow *et al.* 2000).

In view of the reported effects of ROS on the BK_{Ca} channel and the fact that this channel is highly expressed in pregnant human myometrium, we hypothesized (i) that ROS modulate human myometrial contractility, and (ii) that this may occur via mechanisms that involve BK_{Ca} channels. The following study was undertaken to test these hypotheses.

Materials and Methods

Myometrial samples were collected from women undergoing elective non-labour Caesarean section (38–41 weeks gestation) for previous Caesarean delivery, breech presentation or maternal request. Ethical approval was granted by the Southern Derbyshire Ethics Committee and written informed consent was obtained from all patients. Myometrial biopsies were taken from the upper section of the lower segment uterine incision and placed immediately in cold physiological salt solution (PSS) for transport to the laboratory. Samples were stored at 4°C and used within 12 h of collection.

Myometrial samples were dissected free from serosal and decidual tissue and cut into eight longitudinal strips (2 × 2 × 10 mm). Each strip was mounted by means of a stainless steel hook and thread in 10 ml PSS in an organ bath constantly bubbled with a 5% CO₂/95% O₂ gas mixture at 37°C. A passive tension of 2 g was applied to each strip before stimulation with 0.5 nM oxytocin to induce myometrial contractions. Myometrial strips were allowed to equilibrate until stable contractions were observed (approximately 90–120 min) after which the following experiments were performed.

Effect of H₂O₂

H₂O₂ (1 μ M–100 mM) was added to the contracting strips in a cumulative dose-dependent manner every 25 min with or without pre-incubation with the non-specific BK_{Ca} channel blocker tetraethylammonium chloride (TEA; 1 mM) or CAT (10 or 100 μ g/ml).

Effect of the O₂^{•−} anion

O₂^{•−} anion was generated by the reaction of xanthine oxidase (XO) with hypoxanthine (HX). XO was introduced to the baths 5 min before the addition of HX (0.1 or 1 mM). No significant difference was apparent when using either HX concentration, hence subsequent experiments were carried out with 0.1 mM HX. The HX/XO were added at 25 min intervals. Cumulative concentration–response curves with XO were carried out using two concentration ranges: a low range (0, 1, 2, 5, 10 and 20 mU/ml) and a high range (0, 10, 20, 40, 60, 80 and 100 mU/ml), while HX was fixed at 0.1 mM. Due to the observed non-specific effects of using a non-dialysed XO preparation, experiments were repeated with the same concentrations of HX/XO after dialysing XO and carrier against large volumes of PSS overnight at 4°C. Since the enzyme XO was manufactured as a suspension in ammonium sulphate (2.3 M) and sodium salicylate (1 mM), contractile responses were also recorded with the corresponding concentrations of these molecules, without XO, to assess non-specific effects. In order to establish the specificity of ROS action, experiments were carried out by pre-incubating myometrial strips with the antioxidative enzyme SOD (10 or 100 units/ml) or CAT (100 or 1000 μ g/ml) for 30 min in order to quench the production of O₂^{•−} anion and H₂O₂ respectively.

Data acquisition and analysis

Changes in isometric tension, detected by mechanical displacement of force transducers (AD Instruments, Oxford, UK) were digitized and the data collected using the PowerLab system with Chart 4.1 software (AD Instruments). Control contractile responses (% maximal contractility) were calculated from oxytocin-induced contractility data obtained over a 20 min period before addition of test agents. Data for each series of experiments were analysed by the calculation of activity integrals expressed as a function of % maximal contractility at each concentration of drug used. The resulting dose–response curves were compared using Prism 4 software (GraphPad, San Diego, CA, USA). Paired Student's *t*-test or one-way ANOVA was used to assess variability between means. A *P* value < 0.05 was regarded as statistically significant. Results are reported as means $\pm$ S.E.M. for *n* observations.

Drugs and solutions

Oxytocin, XO, HX, SOD, CAT, TEA and H₂O₂ were all purchased from Sigma-Aldrich Co., Ltd. Where applicable all drugs were prepared as stock solutions in PSS and stored frozen (−20°C) until the day of use. The composition of the PSS used in all experiments was as follows: (mM) NaCl 119, CaCl₂·2H₂O 1.6, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.18, MgSO₄·7H₂O 1.17, glucose 5.5 (pH 7.4 when gassed with 5% CO₂/95% O₂).

Results

Myometrial strips mounted for isometric tension recordings exhibited rhythmic, reproducible contractions to stimulation with 0.5 nM oxytocin. Control experiments (with no additional application of drugs) showed little change in either magnitude or frequency of contractions for the duration of the experiment (data not shown).

Effect of H_2O_2

Application of H_2O_2 (10^{-6} – 10^{-1} M) produced a concentration-dependent relaxation of myometrial strips stimulated with oxytocin (Fig. 1, upper panel). A maximal decrease in contractility to $27.2 \pm 4.5\%$ ($n = 7$) with 100 mM H_2O_2 compared with control activity was noted ($P < 0.001$; Fig. 2. Pretreatment of tissue with the free-radical scavenger CAT at 1000 μ g/ml partially inhibited the relaxation produced by H_2O_2 (Fig. 1, lower panel) to a maximum reduction in force of $57.3 \pm 7.8\%$ of control activity ($n = 7$). This effect achieved significance at concentrations of 10^{-4} M and above ($P < 0.05$; Fig. 2). Incubation of tissue strips with lower concentrations of CAT (100 μ g/ml) had no significant effect on contractility ($n = 5$; $P > 0.05$). Pretreatment of tissues with the BK_{Ca} channel blocker, TEA at a concentration of 1 mM did not affect H_2O_2 -mediated relaxation.

Effect of the O_2^- anion

Cumulative addition of non-dialysed XO (0–100 mU) and 0.1 mM HX to produce O_2^- anion, caused a significant dose-dependent decrease in contractility of the myometrial strips (data not shown). This prompted us to perform experiments to investigate the effects of the carriers, ammonium sulphate and sodium salicylate, that formed the XO suspension, the outcomes of which demonstrated a significant effect of carrier molecules on myometrial activity. Hence, experiments were repeated with XO that had been dialysed against PSS. The results show that

Figure 1 Upper panel: application of H_2O_2 (10^{-6} – 10^{-2} M) reduced maximal contractility of the human myometrium in a dose-dependent manner. Lower panel: pre-incubation with 1000 μ g/ml CAT before addition of H_2O_2 prevented the relaxant action of H_2O_2 .

Figure 2 Application of H_2O_2 (●) reduced maximal contractility of the human myometrium in a dose-dependent manner. Pre-incubation with 1000 μ g/ml CAT (▲) before addition of H_2O_2 reduced the relaxant effect of the latter. Means $\pm$ S.E.M. * $P < 0.05$; ** $P < 0.01$. $n = 7$.

HX/XO dose-dependently reduced myometrial contractility. Figure 3 illustrates a representative trace showing the effect of SOD/CAT pretreatment on subsequent HX/XO addition. A maximal reduction to $23.8 \pm 4.2\%$ of control ($n = 8$; $P < 0.001$; Fig. 4) at the highest concentration of XO tested (100 mU/ml), while HX was held constant at 0.1 mM. Addition of SOD (100 units/ml) and CAT (100 μ g/ml) to the bath caused a significant reduction in the ability of HX/XO to relax myometrial strips with a $62.2 \pm 6.5\%$ inhibition in force compared with control activity ($n = 8$; $P < 0.05$; Fig. 4).

In preliminary experiments, we observed that a single application of XO/HX was sufficient to fully inhibit myometrial contractility over 1 h. This phenomenon was examined further by testing the effect of a single dose of 100 mU XO/0.1 mM HX (undialysed and dialysed) on frequency of myometrial contractions. Addition of undialysed 100 mU XO/0.1 mM HX abolished contractions by 1 h ($n = 4$; $P > 0.05$) compared with control strips (data not shown). However, dialysed HX/XO had no significant

Figure 3 Upper panel: application of HX/XO reduced contractility of the human myometrium in a concentration-dependent manner. Small arrows indicate addition of 0.1 mM HX 5 min before incremental increases in XO (dashed lines). Lower panel: pre-incubation with 1000 μ g/ml CAT and 100 units/ml SOD prevented the relaxant effect of H_2O_2 .

Figure 4 The effect of HX/XO (O_2^- -generating system) on oxytocin-induced myometrial contractility. The graph depicts the decrease in myometrial contractility due to the effects of HX/XO (●). Pre-incubation with SOD/CAT before HX/XO (▲) reduced inhibition compared with H_2O_2 alone. Means $\pm$ S.E.M. * $P < 0.05$. $n = 8$.

effect on % frequency compared with controls ($n = 5$; $P > 0.05$).

Dialysed carrier alone or in the presence of SOD/CAT had no significant effect on myometrial contractility, reducing it respectively to 84.1 ± 6.8 and $78.6 \pm 3.8\%$ of control over a 3 h recording period ($n = 6$; $P > 0.05$; Fig. 5). Nor was this effect significantly different from that with PSS replacing dialysed carrier solution (Fig. 5).

Discussion

Spontaneous vaginal delivery requires strong, coordinated contractions of the uterus to propel the fetus through the birth canal. In some cases, contractions may cause compression of blood vessels supplying the uterus. The episodic nature of this process is likely to trigger a chain of reperfusion–ischaemia events which result in the generation of ROS. Indeed, the human myometrium produces ROS, levels of which are elevated during labour (Zyrianov *et al.* 2003). In addition, the human myometrium is endowed with enzymatic scavenging systems (SOD, CAT,

Figure 5 The effect of dialysed carrier (●), carrier + SOD/CAT (▲), PSS alone (○) and PSS + SOD/CAT (△) on contractility with time. There was no significant difference between any of the treatments. $n = 4$.

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glutathione peroxidase) that minimize the potentially destructive effects of ROS (Telfer *et al.* 1997, Matharoo-Ball & Khan 2003).

In this study we have explored the hypothesis that ROS modify the contractility of the human myometrium and that this may contribute to the disruption of regular coordinated myometrial contractions during the process of labour. To the best of our knowledge this is the first study to examine the direct effects of O_2^- anion and H_2O_2 on the contractile properties of human myometrium. Our results provide evidence that H_2O_2 caused a diminution in contractions of non-labour human myometrium, which was considerably reduced after addition of CAT. Paradoxically, Cherouny *et al.* (1989) using pregnant rat myometrial segments illustrated that H_2O_2 enhanced contractility and that this effect occurred concomitantly with increased prostaglandin- $F_{2\alpha}$ and $-E_2$ release. The disparity between our study and that of Cherouny *et al.* (1989) could be explained by species differences in the control of myometrial function, gestation and parturition.

There is mounting evidence that H_2O_2 may exert its effects through many cellular targets that include membrane ion channels. Raised $[Ca^{2+}]_i$ levels triggering cell death have been linked to H_2O_2 acting on a non-selective cation channel in rat CRI-G1 cells (Herson *et al.* 1999). However, elevated $[Ca^{2+}]_i$ levels, elicited by H_2O_2 , may also activate BK_{Ca} channels to promote relaxation as shown in canine trachealis (Janssen *et al.* 2000). BK_{Ca} channels are expressed at high density in myometrial cells and are important effector molecules that mediate relaxation. Our observation that pretreatment of myometrial strips with 1 mM TEA, an extracellular concentration that specifically blocks BK_{Ca} channels (Khan *et al.* 1993), did not overcome the H_2O_2 -induced relaxation hints at the involvement of other mechanisms. In canine trachealis, relaxations triggered by H_2O_2 and $OH^\bullet$ are thought to act via several different ion channel subtypes (Janssen *et al.* 2000). It is possible therefore, that the H_2O_2 effects uncovered in our study may similarly involve more than one particular ROS acting to modify uterine function.

Experimental hypoxia is linked to a reduction in the force of myometrial contractions but it is far from clear how and why uterine contractions wane during some labours. While undoubtedly due in part to the excessive energy demands of the uterus to support labour as well as the power of the contractions, we hypothesized that cellular damage to contractile elements, due to ROS activity at muscle cells, would interfere with this cascade. Our own observations suggest that short hypoxic insults reversibly reduce the contractile force of human myometrial strips. However, longer exposure to hypoxia (> 40 min) resulted in abolition of myometrial contractions (A Y Warren & R N Khan, unpublished observations) consistent with our data revealing a decrease in uterine contractility with O_2^- anion. This finding is also in agreement with a number of studies reporting altered contractile properties in canine trachealis (Janssen *et al.* 2000), guinea-pig trachea (Matyas

et al. 2002) and rat diaphragm (Callahan *et al.* 2001) smooth muscle. It is significant that the relaxation produced by O_2^- anion with dialysed XO was much less than that evident using non-dialysed XO and emphasized the importance of proper controls. In contrast, Masumoto *et al.* (1990) reported O_2^- induced increases in $[Ca^{2+}]_i$ in human myometrium. They postulated that this would translate to enhanced myometrial contractility. However, these opposing observations may be explained by the different techniques employed in the two studies where Masumoto *et al.* (1990) used digital microscopy to evaluate the changes in $[Ca^{2+}]_i$ concentrations while the present study investigated the effects of HX/XO directly on myometrial contractions. Masumoto *et al.* (1990) also utilized enzymatically dispersed cells, hence it is difficult to simply extend these findings to tissue strips that are likely to have intact signalling pathways. It is also feasible that endogenous O_2^- dismutates spontaneously to H_2O_2 . Unlike impermeant O_2^- anion, H_2O_2 crosses biological membranes with relative ease, which may mean that the actions of H_2O_2 on isolated cells are probably more rapid. This would lend support to our finding that both H_2O_2 and O_2^- impair myometrial function. The lack of effect of SOD/CAT to contracting tissue strips without HX/XO suggests that the antioxidative capacity in our myometrial preparations is highly effective at removing ROS. This is supported by our findings that human myometrium expresses high levels of Cu/Zn-SOD and CAT (Matharoo-Ball & Khan 2003).

When ROS production exceeds the scavenging capacity of myometrial antioxidant defences, oxidative stress may arise with consequent cellular dysfunction and tissue damage. The severity of muscle fatigue during prolonged labour is exacerbated by the actions of ROS. One of the deleterious outcomes of ROS damage, lipid peroxidation, results in altered membrane fluidity and local membrane disruption of the lipid bilayer that may perturb native signalling networks, thereby adversely affecting muscle function. An improved understanding of the physiological pathways that modulate ROS-mediated effects on uterine contractility will help define the complex processes that underlie parturition (term and preterm). This may lead to new scientific approaches to manage dysfunctional labours, possibly by limiting ROS production through stimulation of enzymatic or non-enzymatic antioxidative pathways.

In conclusion, the effects of H_2O_2 and O_2^- anion on myometrial contractility highlight the complex interactions that exist between ROS signalling and control of muscle function in the pregnant human myometrium. Deciphering the pathways through which these oxidants operate may raise novel therapeutic opportunities in obstetrics.

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