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Vazoactive Effects of Oxidative Stress Elicited by Hydrogen Peroxide in the Human Umbilical Artery: An *in Vitro* Study

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ABSTRACT

The vasoactive effects of oxidative stress induced by hydrogen peroxide (H_2O_2) on human umbilical artery strips as well as the possible mechanisms involved are studied. Contraction responses to cumulative H_2O_2 (10^{-7} M - 3×10^{-2} M) in endothelium intact and denuded umbilical arteries and responses to cumulative H_2O_2 after incubation with L-NAME (10^{-4} M) ($n = 8$), indomethacin (10^{-5} M) ($n = 8$) and verapamil (10^{-6}) ($n = 8$) were recorded. Responses elicited with cumulative H_2O_2 in Ca^{2+} free extracellular medium and the responses to cumulative Ca^{2+} (10^{-4} M - 2×10^{-3} M) after H_2O_2 (10^{-3} M) induced contraction were also studied. The E_{max} for each experiment was calculated. $p < 0.05$ was considered as significant. H_2O_2 elicited contraction was greater in endothelium denuded artery strips compared to endothelium intact strips ($p < 0.05$). Compared to control, incubation with L-NAME significantly augmented ($p < 0.05$), while verapamil and indomethacin inhibited the contractions elicited by cumulative H_2O_2 ($p < 0.05$). Ca^{2+} free extracellular medium caused decreases in cumulative H_2O_2 elicited contractions and cumulative Ca^{2+} caused concentration dependent increases in the contraction caused by a single bolus of H_2O_2 ($p < 0.05$). Exposure to H_2O_2 causes concentration-dependent constriction in human umbilical arteries. The presence of the endothelium and NOS enzyme activation influences the H_2O_2 responses. Removal of the endothelium increases the H_2O_2 elicited contractions more than incubation with L-NAME suggesting beside NO, other endothelial vasodilators are also involved in vascular tonus of the umbilical arteries. Both intracellular and extracellular Ca^{2+} ions and constrictor cyclooxygenase metabolites play a role in the contractile responses elicited by H_2O_2 in human umbilical arteries.

Keywords: *Umbilical Arteries*, *Hydrogen Peroxide*, *Indomethacin*, *L-NAME*, *Oxidative Stress*, *Pre-Eclampsia*, *Reactive Oxygen Species*, *Verapamil*

1. Introduction

Pre-eclampsia (PE) is a major cause of fetal growth restriction and perinatal complications. In PE, there is increased resistance to placental circulation which leads to reduced uteroplacental blood flow followed by placental dysfunction and intrauterine fetal growth restriction [1,2]. Umbilical blood vessels are not innervated therefore the control of umbilical blood flow depends entirely on vasoreactive substances either released locally or presented in the circulation [3]. Pregnancy is a state of oxidative stress, characterized by the placental production of reactive oxygen species (ROS) including superoxide and hydrogen peroxide (H_2O_2) [4,5]. It is considered that during normal pregnancy, the rate of production of ROS is offset by their elimination by abundant antioxidant defenses. However in PE and preterm labor, due to excessive oxidative stress and lipid peroxidation (LPO) ROS overpowers antioxidant defenses, leading to reduction of uteroplacental blood flow [5,6]. H_2O_2 is a powerful by product of LPO and is used as a model of oxidative stress. H_2O_2 easily crosses cell membranes and lead to cellular oxidative damage. LPO has been studied intensively over decades and remains to be a hot topic in biological research but still there is little information on the effects of LPO on human umbilical arteries [7-9]. This *in vitro* study was designed to assess the effects of H_2O_2 used as a model of oxidative stress on human umbilical arteries as well as the possible mechanisms involved.

2. Materials and Methods

The University Human Ethics Committee approved this study. All umbilical cords used in the experiments were remnant tissues, which would have otherwise been discarded.

2.1. Sample Collection

After maternal consent, human umbilical cords were collected from healthy full-term normal deliveries. After delivery, the umbilical cord was clamped at both placental and fetal ends. An untouched 15 - 20 cm long segment of the umbilical cord was taken from the placental side within 10 min of delivery and placed in cold Krebs-Henseleit solution for transport to the laboratory.

2.2. Blood Vessel Preparation

Umbilical arteries were separated from the surroundings in warm modified Krebs-Henseleit solution. The isolated artery was cut spirally to form 2 - 3 mm wide and 15 - 20 mm long strips. The strips were suspended between two stainless steel hooks in organ baths (10 ml) containing Krebs-Henseleit buffer maintained at 37˚C. One hook was anchored onto the organ bath and the other was connected to a movable transducer (Model FT 03, Grass Instrument Co. MA, USA) and a polygraph (Model 7, Grass Instrument Co. MA, USA) for measurement and recording of changes in isometric tension.

2.3. Experimental Protocols

Protocols were conducted with endothelium intact artery strips except for protocol 1 which used both endothelium intact and endothelium denuded artery strips. Endothelium removal was done by gently denuding the endothelium of the artery with cotton swabs. The integrity of the endothelium was tested by, first pre-contracting the denuded strips with serotonin (10^{-4} M), and then adding acetylcholine (10^{-6} M) before each experiment. Removal of the endothelium was confirmed if the vessels contracted in response to acetylcholine.

Strips were aerated with a gas mixture of 95% O_2:5% CO_2 throughout the experiment. Strips were initially placed under a resting tension of 1 g and were allowed to equilibrate for one hour. During this period the bath solution was changed every 15 minutes and the resting tension was readjusted to the 1 g level. Following the washout period, the initial control contraction of the strips to serotonin (10^{-4} M) was recorded. The strips were washed again with the buffer solution and allowed to rest. After an equilibrium period, the following procedures were conducted at 37˚C:

Protocol 1: to determine the role of the endothelium on the reactivity of the human umbilical artery to H_2O_2, umbilical artery strips both with intact endothelium (n = 8) and denuded endothelium (n = 8) were used. The strips precontracted with serotonin (10^{-4} M) were subjected to cumulative doses of H_2O_2 (10^{-7} M - 3×10^{-2} M) at resting tension and concentration-response curves were obtained. Further protocols were conducted with endothelium intact artery strips.

Protocol 2: to determine the role of nitric oxide (NO) in the mechanism of H_2O_2 elicited contractions; umbilical artery strips precontracted with serotonin (10^{-4} M) were incubated for 20 minutes with a NO synthase inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME) (10^{-4} M) (n = 8) After this incubation, concentration-response curves were obtained to cumulative H_2O_2 (10^{-7} M - 3×10^{-2} M).

Protocol 3: to determine the role of prostanoids in the mechanism of H_2O_2 elicited contractions; umbilical artery strips precontracted with serotonin (10^{-4} M) were incubated for 20 minutes with a cyclooxygenase inhibitor, indomethacin (10^{-5} M) (n = 8). After this incubation, concentration-response curves were obtained to cumulative H_2O_2 (10^{-7} M - 3×10^{-2} M).

Protocol 4: to determine the role of Ca^{2+} channels in the mechanism of H_2O_2 elicited contractions; umbilicalk artery strips precontracted with serotonin (10^{-4} M) were incubated for 20 minutes with a Ca^{2+} channel blocker, verapamil (10^{-6} M) (n = 8). After this incubation, concentration-response curves were obtained to cumulative H_2O_2 (10^{-7} M - 3×10^{-2} M).

Protocol 5: the effects of Ca^{2+} on H_2O_2 elicited responses were studied in another group of umbilical arteries (n = 8). The artery strips were allowed to rest in modified Ca^{2+} free Krebs-Henseleit solution containing 1 mM of ethyleneglycol-bis-(β-aminoethyl ether) N'tetraacetic acid (EGTA) for 60 min, which was changed every 15 min. Cumulative H_2O_2 (10^{-7} M - 3×10^{-2} M) was added to the organ bath and concentration-response curves were obtained. In a different group of umbilical strips (n = 8), the strips were allowed to rest in modified Ca^{2+} free Krebs-Henseleit solution containing 1mM of EGTA for 60 min, which was changed every 15 min. After obtaining a contraction curve with a bolus of H_2O_2 (10^{-3} M), cumulative Ca^{2+} (10^{-4} M - 2×10^{-3} M) was added to the organ bath and concentration-response curves were obtained.

2.4. Materials

H_2O_2, magnesium sulphate ($MgSO_4$), potassium hydrogen phosphate (KH_2PO_4) sodium bicarbonate ($NaHCO_3$), potassium chloride (KCl), sodium chloride (NaCl), and calcium chloride ($CaCl_2$) were obtained from Merck

(Merck KGaA, Darmstadt, Germany) and serotonin hydrochloride, L-NAME, indomethacin, verapamil and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Krebs-Henseleit solution and modified Krebs-Henseleit without calcium were prepared in the laboratory with compositions of (in mM) NaCl 119; KCl 4.7; $MgSO_4$ 1.5; KH_2PO_4 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 25; glucose 11, and NaCl 119; KCl 4.7; $MgSO_4$ 1.5; KH_2PO_4 1.2; $NaHCO_3$ 25; glucose 11, EGTA 1 respectively. All agents were dissolved in distilled water.

2.5. Data and Statistical Analysis

The contraction is expressed as percentage (%) of the contractile level that was induced by serotonin. The E_{max} (% of maximum contraction) in each group and pD_2 (the negative logarithm of the concentration which elicits 50% contraction) for groups in which $E_{max} > 50\%$ were calculated. All results are expressed as the mean ± standard deviation of mean and n denotes the number of human umbilical cords which the arterial strips were obtained. Analysis of variance (ANOVA) and Tukey's HSD tests were used were appropriate to determine the differences between the percentage values using a computer statistical package (SPSS, Chicago, IL, USA). $p < 0.05$ was considered as significant.

3. Results

3.1. The Effect of Endothelium on H_2O_2 Elicited Contractions

H_2O_2 (10^{-7} M - 3 × 10^{-2} M) elicited concentration dependent contraction in isolated human umbilical artery strips both with (E_{max} = 63.5 ± 3.7, pD_2 = 3.08 ± 0.1) and without (E_{max} = 101.8 ± 9.6, pD_2 = 4.10 ± 0.26) endothelium. There were significant differences among these strips in terms of E_{max} and pD_2 values with significantly larger contractions in the endothelium denuded strips ($p < 0.05$) (**Figure 1**).

3.2. The Effect of L-NAME, Indomethacin and Verapamil Incubation on H_2O_2 Elicited Contractions

Compared to control (E_{max} = 63.5 ± 3.7, pD_2 = 3.08 ± 0.0), incubation with L-NAME significantly augmented (E_{max} = 91.8 ± 8.3, pD_2 = 3.74 ± 0.2) ($p < 0.05$), while verapamil (E_{max} = 25.1 ± 3.3) and indomethacin (E_{max} = 14.1 ± 3.4) significantly inhibited the contractions elicited by cumulative H_2O_2 ($p < 0.05$) (**Figure 2**).

3.3. The Effect of Ca^{2+} on H_2O_2 Elicited Contractions

When compared with the maximum contraction responses with Krebs-Henseleit solution (E_{max} = 63.5 ± 3.7, pD_2 = 3.08 ± 0.0), providing a Ca^{2+} free extracellular medium caused significant decreases in cumulative H_2O_2 elicited contractions (E_{max} = 38.7 ± 5.8) ($p < 0.05$) (**Figure 3**). After resting in modified Ca^{2+} free Krebs-Henseleit solution, cumulative Ca^{2+} (10^{-4} M - 2 × 10^{-3} M) caused significant concentration dependent increases (E_{max} = 98.5 ± 6.1) in the contraction caused by a single bolus of H_2O_2 (E_{max} = 23.3 ± 3.3) ($p < 0.05$) (**Figure 4**).

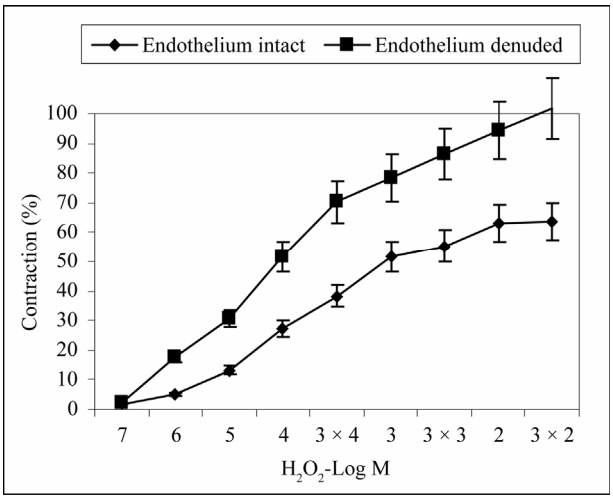

Figure 1. Concentration-response curves for cumulative H_2O_2 (10^{-7} M - 3 × 10^{-2} M) on isolated human umbilical artery strips with and without endothelium. Data expressed as the percentage of the control contractile response elicited by 10^{-4} M of serotonin. Mean ± SD (n = 8).

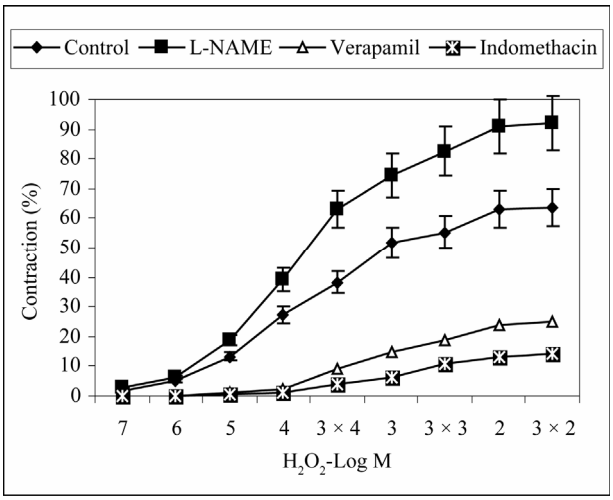

Figure 2. Concentration-response curves for cumulative H_2O_2 (10^{-7} M - 3 × 10^{-2} M) on isolated human umbilical artery strips with endothelium after incubation with L-NAME (10^{-4} M), verapamil (10^{-6} M) and indomethacin (10^{-5} M). Data expressed as the percentage of the control contractile response elicited by 10^{-4} M of serotonin. Mean ± SD (n = 8).

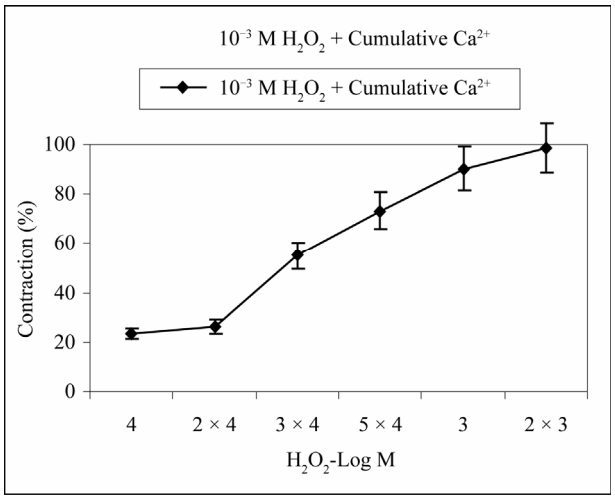

Figure 3. Concentration-response curves for cumulative Ca^{2+} (10^{-4} M - 2 × 10^{-3} M) on H_2O_2 (10^{-3} M) induced constriction of human umbilical arteries. Data expressed as the percentage of the control contractile response elicited by 10^{-4} M of serotonin. Mean ± SD (n = 8).

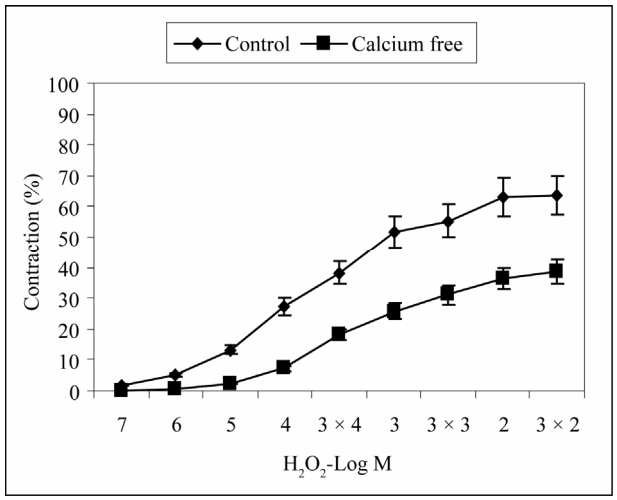

Figure 4. Concentration-response curves for cumulative H_2O_2 (10^{-7} M - 3 × 10^{-2} M) on isolated human umbilical artery strips in Krebs-Henseleit solution and modified Krebs-Henseleit without calcium. Data expressed as the percentage of the control contractile response elicited by 10^{-4} M of serotonin. Mean ± SD (n = 8).

4. Discussion

The present study demonstrates that *in vitro* experimental model of oxidative stress elicited by H_2O_2 causes concentration dependent vasoconstriction in human umbilical arteries both with and without endothelium. Both intracellular and extracellular Ca^{2+} ions and cyclooxygenase enzyme activation play a role in the contractile responses elicited by H_2O_2 in human umbilical arteries. The presence of the endothelium and NOS enzyme activation influences the H_2O_2 responses.

The known vasoactive substances responsible for the control of umbilical flow include local vasoconstrictors such as endothelin-1, thromboxane A2 and prostaglandin F2α and also vasodilators such as prostacyclin (PGI2), nitric oxide (NO) and end8othelium derived hyperpolarizing factor (EDHF) [10,11]. It is known that under oxidative stress conditions such as pre-eclampsia placental vascular resistance increases [12,13]. The mechanisms by which ROS cause vasoconstriction are incompletely understood. At a vascular level, endothelial cells are a target for, and a source of, H_2O_2. Previous work with different species and vascular structures yielded conflicting results. Similar to our study, H_2O_2 caused vasoconstriction in human umbilical artery [14,15], pulmonary artery [16] and rat aorta [17]. Contrary to these results, H_2O_2 caused vasodilation in rabbit [18], rat mesenteric artery [19], guinea-pig aorta [20] and porcine coronary artery [21]. The above results lead us to think that the vasoactive properties of H_2O_2 vary with species, tissue and experimental conditions [20,22]. Therefore isolated human placental arteries and veins should be considered most suitable for *in-vitro* PE models.

In the present study our data suggest that cumulative H_2O_2 causes more potent vasoconstriction in endothelium denuded human umbilical arteries. This effect may be attributed to the loss of vasodilators produced by the endothelium such as NO, EDHF and PGI_2. Similar results have been shown previously in the rat aorta [17,22]. Rodriguez-Martinez *et al*. [17] have demonstrated that oxygen-derived free radicals are involved in the contractile effects of H_2O_2; endothelium protects against oxidative injury caused by H_2O_2 in smooth muscle cells, endothelial NO has a protective role on the contractile effect induced by H_2O_2 in normotensive rats; and this protective role of endothelial NO is lost under oxidative stress such as hypertension. The role of ROS and NO in the pathogenesis of pre-eclampsia using human umbilical vein endothelial cell cultures, have been investigated by Matsubara *et al*. [9] in normal and pre-eclampsia patients. They argued that endothelial dysfunction during PE possibly results from the inactivation of NO by superoxide ions. This is supported by our results, in which umbilical artery strips incubated with L-NAME, which is a NO synthase inhibitor, significantly increased the contraction elicited by H_2O_2. Furthermore, complete endothelial removal resulted in higher vasoconstriction than the L-NAME incubated human umbilical arteries. This implies that besides NO, other endothelial factors such as EDHF and PGI2 may be involved in the process. Together with previous literature, these results lead us to think that NO

acts as a negative modulator against H_2O_2 elicited contractions in placental arteries and also may be protective against oxidative insult [17,19,22].

Lelung *et al.* [3] have demonstrated that sodium nitroprusside, an exogenous source of NO, only elicits small significant relaxations in isolated human umbilical arteries. They are in the opinion that NO might not be the major mediator responsible for vasodilation in umbilical arteries. Previous work also suggests that, instead of NO prostacyclin was the main factor mediating the endothelium dependent relaxation in human umbilical arteries [10,14,23]. Klockenbush *et al*. [24] have suggested that rather than NO, prostacyclin plays a major role in the vasoreactivity of umbilical and fetal circulation. H_2O_2 elicited vasoreactivity can be relaxation or constriction depending on the basal tonus of isolated pulmonary arteries, and these effects are mediated by phospholipase A2 activation leading to prostacyclin or thromboxane A2 release [25]. In the present study the H_2O_2 elicited contraction in umbilical arteries were significantly inhibited in the presence of indomethacin, a cyclooxygenase enzyme inhibitor. These data suggest that increased constrictor cyclooxygenase metabolites as a result of increased arachidonic acid metabolism partially mediate the constrictive effects of H_2O_2 in the vascular smooth muscle of human umbilical arteries. Previous research shows that prostaglandin H2 and more possibly thromboxane A2 are involved in the contractile response [26, 27]. These findings are in accordance with studies which, showed that H_2O_2 increases prostaglandin F2α and thromboxane A2 in rat aorta smooth muscle [28,29].

Ca^{2+} is essential for the contraction of smooth muscles and it is believed that intracellular Ca^{2+} homeostasis plays a major role in antioxidant activity [30-33]. The harmful effects of ROS can influence the ion channels or ion pumps, which maintain low Ca^{2+} levels under normal conditions [33,34]. It is known from previous work that in different tissues H_2O_2 can increase intracellular Ca^{2+} by promoting mobilization of Ca^{2+} and Ca^{2+} influx [31,33, 34]. In isolated rat cardiomyocytes Gen *et al*. [33] have shown that H_2O_2 increases intracellular Ca^{2+} in a dose dependent manner. In our study we have found that H_2O_2 induced contractions increase in a dose dependent pattern when cumulative Ca^{2+} was added after a single bolus of H_2O_2 in human umbilical arteries.

It has been reported that NO modulates Ca^{2+}-channel activity in vascular smooth muscle and induces relaxation [35]. Incubation with verapamil, a Ca^{2+}-channel antagonist significantly decreased but did not totally block H_2O_2 induced contractions. Sotnikova [22] and Yang *et al*. [36] have reported similar results in rat aorta with calcium antagonists. In the present study, both incubation with verapamil and resting the arteries in Ca^{2+}-free medium attenuated but did not prevent H_2O_2 induced contractions. These findings suggest that both intracellular and extracellular Ca^{2+} mediate these contractions. It has been suggested previously that the decrease caused by ROS on membrane resistance may depolarize the cells and thus activate voltage sensitive Ca^{2+} channels and lead to an increase in intracellular Ca^{2+} [31,32]. Also, Ca^{2+} channel blockers are thought to be ineffective in blocking mobilization of Ca^{2+} from intracellular stores but effectively block the influx of extracellular Ca^{2+} via the L type Ca^{2+} channels [33].

5. Conclusions

In conclusion, in this *in-vitro* model for oxidative stress we have demonstrated that exposure to H_2O_2 causes concentration-dependent constriction in human umbilical arteries. Removal of the endothelium increased the H_2O_2 elicited contractions more than incubation with L-NAME suggesting beside NO, other endothelial vasodilators are also involved in vascular tonus of the umbilical arteries. Inhibition of H_2O_2 elicited contractions with indomethacin suggests that increased constrictor cyclooxygenase metabolites partially mediate the constrictive effects of H_2O_2 in the vascular smooth muscle of human umbilical arteries. Incubation with verapamil, and Ca^{2+} free medium significantly decreased but did not totally block H_2O_2 induced contractions suggesting both intracellular and extracellular Ca^{2+} involvement in H_2O_2 elicited contractions.

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