Pharmacological Reports
2011, 63, 1019–1028
ISSN 1734-1140

Effect of indometacin pretreatment on protamine sulfate-mediated relaxation of the isolated rat uterus: the role of the antioxidative defense system

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Abstract:
Previous results in this laboratory indicate that protamine sulfate (PS) evokes dose-dependent relaxation of both spontaneous and calcium ion-induced uterus activity mediated predominantly by potassium channels and, to a small extent, *via* β-adrenergic receptors or nitric oxide (NO)-dependent pathways. Indometacin is a nonselective inhibitor of cyclooxygenase (COX 1 and COX 2) that has the ability to delay premature labor by reducing uterine contractions through the inhibition of prostanglandin synthesis in the uterus. This study investigates the effects of indometacin (0.1 and 1 μg/ml) pretreatment on the PS-induced relaxation of isolated uterine smooth muscle.
Indometacin pretreatment *per se* did not change the activity of the uteri. However, indometacin significantly increased PS-induced relaxation of spontaneous uterine contractions. Indometacin pretreatment significantly decreased the magnitude and slope of PS-induced relaxation of calcium ion-induced uterine contractions. Indometacin pretreatment increased CuZnSOD activity and slightly increased GR activity during spontaneous uterine contractions when compared to PS alone. In calcium ion-induced contractions, indometacin pretreatment increased CuZnSOD, GSH-Px and GR activities. These results suggest that, in addition to its COX inhibitory effects, indometacin influences the effects of PS. Therefore, it is possible that indometacin regulates diverse cell functions *via* its association with lipid membranes by altering micro-environments within the membranes. The above-mentioned processes appear to be partly mediated by redox processes involving ROS, lipid peroxides and antioxidant enzymes. The extent of the PS-mediated effect was different in spontaneous *versus* calcium ion-induced active uteri.

Key words:
indometacin, protmine sulfate, CuZnSOD, GSH-Px, GR, uterus

Abbreviations: AD – anti-oxidant, ADS – antioxidant defence system, AR – adrenoceptor; ATP – adenosine triphosphate, BBM – brush border membranes, BK_{Ca} – large conductance calcium-activated potassium channel, cAMP – cyclic adenosine monophosphate, CAT – catalase, cGMP – cyclic guanosine monophosphate, COX – cyclooxygenase, CuZnSOD – copper-zinc

superoxide dismutase, DTT – dithiothreitol, GR – glutathione-reductase, GSH-Px – glutathione-peroxidase, MnSOD – manganese superoxide dismutase, PS – protamine sulfate, ROS – reactive oxygen species, SLP – surfactant-like particles

Introduction

Protamine sulfate (PS) is a mixture of polyamines isolated from the sperm of Clupeidae or Salmonidae fish families and is used clinically to reverse heparin overdose [30]. Its precise mode of action remains unclear [22]. In clinical use, PS has demonstrated systemic and multiple physiological effects *via* modulating signal transduction pathways. It has been proposed that PS acts, at least in part, through its interaction with calcium ion influxes and/or calcium ion release from intracellular stores [10, 25]. Our previous results demonstrate PS dose-dependent relaxation of smooth muscles from both vascular and uterine tissues [26, 27]. Results obtained in the isolated uterus indicate that PS action is mediated *via* K^+ channels [24, 25]. In spontaneously active uteri, the large conductance calcium-activated potassium channel (BK_{Ca}) and two delayed rectifier K^+ currents play significant roles in mediating dose-dependant PS-mediated relaxation. When smooth muscle contractions are calcium-induced, all three types of potassium channels play a crucial role in PS dose-dependant relaxation. However, the uterus is rich in different types of receptors. Furthermore, the regulation of uterine relaxation is poorly understood, as is the contribution of different types of receptors and channels for the regulation of myometrial contractility. Studies of myometrial tissue and other types of smooth muscle have uncovered a number of receptors, ion channels and regulatory proteins that are likely to be involved [18].

Indometacin is a non-selective inhibitor of cyclooxygenase that affects the contractility of smooth muscles. It can increase [12, 15, 29] or decrease [8, 16, 17, 34–37] the effects of vasoconstriction of vascular smooth muscles. In addition, prostaglandins cause uterine contractions in pregnant women [23]. Inhibitors of prostaglandin synthesis such as indometacin are able to delay premature labor by reducing uterine contractions through the inhibition of prostanglandin synthesis in the uterus [11, 41]. Therefore, we investigated the influence of indometacin pretreatment on PS-dependant uterine relaxation.

Sivalingam et al. [32] have shown that indometacin can induce oxidative stress and mitochondrial dysfunction, both of which have been implicated in the pathogenesis of indometacin-induced enteropathy. First, indometacin-induced oxidative stress and mitochondrial dysfunction have been reported in the small intestines of indometacin-treated rats. Second, significant changes in some mitochondria lipids in the mitochondria, brush border membranes (BBM) and surfactant-like particles (SLP) have been observed in response to indometacin treatment. Third, indometacin has been observed to uncouple oxidative phosphorylation at low concentrations and inhibit respiration at high concentrations in isolated liver mitochondria from rats, mice and humans. The responses of rat, mouse and human mitochondria to indometacin were generally similar [14]. The uterus' role as a reproductive organ is primarily accomplished by contraction and relaxation. Various steps of the contractile process have proven to be susceptible to redox modulation. The growing list of redox-sensitive receptors and kinases in uterine smooth muscle includes myosin light chain kinase, which can be modified by changes in free thiol groups [7]. The control of reactive oxygen species (ROS) provided by antioxidant (AOD) enzymes (superoxide dismutase – SOD; catalase – CAT; glutathione peroxidase – GSH-Px and glutathione reductase – GR) regulates, at least in part, cellular redox states within narrow physiological limits and may be an important regulatory mechanism for various cellular functions. Furthermore, uterine activity is influenced by hydrogen peroxide (H_2O_2), the effect of which is modulated by antioxidant enzymes [3]. Therefore, we sought to elucidate the effect(s) of indometacin pretreatment on PS dose-dependant relaxation by assessing the resulting activities of antioxidant enzymes in uterine smooth muscle.

Materials and Methods

Experimental system

All protocols for handling rats were approved by the local ethics committee for animal experimentation, which strictly follows international regulations. Animals were kept at 22°C, housed 3 per cage and fed *ad libitum*. Isolated uteri from virgin Wistar rats (200–250 g) in estrous, which was determined by examination of a daily vaginal lavage [20], were used.

Reagents

Protamine sulfate (PS) was supplied by Galenika a.d. (Belgrade, Serbia). Indometacin was purchased from Sigma-Aldrich (St. Louis, MO, USA). All drugs were dissolved in ultra-pure water. Salts for De Jalon's solution were obtained from Zorka Pharma (Sabac, Serbia), Merck and Centrohem d.o.o. (Stara Pazova, Serbia).

Isolated organ bath studies

All rats were sacrificed by cervical dislocation. The uterine horns were rapidly excised, carefully cleaned of surrounding connective tissue and mounted vertically in a 10 ml volume organ bath containing De Jalon's solution (NaCl 154 mM, KCl 5.6 mM, $CaCl_2 \times 2H_2O$ 0.41 mM, $NaHCO_3$ 5.9 mM and glucose 2.8 mM), under 1 g tension, aerated with 95% oxygen and 5% carbon dioxide at 37°C. After an equilibration period (of about 30 min), when uteri achieved stable contractions (spontaneous or calcium ion-induced), indometacin (0.1 and 1 μg/ml) was added prior to PS. After 10 min, increasing concentrations of PS were added until the total cessation of contractions was observed.

Myometrial tension was recorded isometrically with a TSZ-04-E isolated organ bath and transducer (Experimetria, Budapest, Hungary).

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

Determination of antioxidant 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). SOD activities were determined by the adrenaline method [21], in which one unit of activity is defined as the amount of enzyme necessary to decrease the rate of adrenalin autooxidation by 50% at pH 10.2. Manganese SOD (MnSOD) activity was determined by incubating the samples with 8 mM KCN. CuZn-SOD activity was calculated as the difference between total SOD and MnSOD activities. The activity of CAT was determined by the rate of H_2O_2 disappearance measured at 240 nm, according to Claiborne [6]. 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 GSH-Px was determined by the GSH-dependent reduction of *t*-butyl hydroperoxide using a modification of the assay described by Paglia and Valentine [28]. One unit of GSH-Px activity is defined as the amount required to oxidize 1 nM NADPH per min at 25°C and pH 7.0. GR activity was determined using the method of Glatzle et al. [9], which is based on NADPH oxidation concomitant with GSH reduction. One unit of GR activity is defined as the oxidation of 1 nM NADPH per min at 25°C and pH 7.4. All enzyme activities are expressed as units/mg protein.

Data analysis and statistical procedures

Statistical analyses (descriptive statistics, analysis of variance (ANOVA) and F-test) were performed according to the protocols described by Hinkle et al. [13] and Manley [19] using Statistical Analysis Software, version 9.1.3 (SAS Institute Inc., NC, USA). Effects of treatments on uterine contractions were calculated as percentages for control, untreated and contracting conditions. Each data value is expressed as the mean ± SEM. Differences between groups were analyzed by two-way ANOVA using treatment and dose as factors and were considered statistically significant when $p < 0.05$. Dose-response curves were sigmoidal in shape and fitted to Boltzmann functions (the concentration axis was linear), and the PS concentration required for half-maximal effect (EC_{50}) was calculated. Sigmoid curves were compared using the F-test. EC_{50} values were compared using one-way ANOVA followed by the *post-hoc* Newman-Keuls test for multiple comparisons (significance: $p < 0.05$). The activities of antioxidant enzymes were compared using one-way ANOVA followed by the Tukey's HSD *post-hoc* test (significance: $p < 0.05$).

Results

PS caused dose-dependent relaxation of spontaneously active uteri (two-way ANOVA, $p < 0.001$, $n = 7$) (Fig. 3 and Fig. 1A). Indometacin pre-treatment (0.1 and 1 μg/ml) increased PS-induced relaxation (two-way ANOVA, $p < 0.001$, $n = 7$) (Fig. 3 and Fig. 1B, 1C),

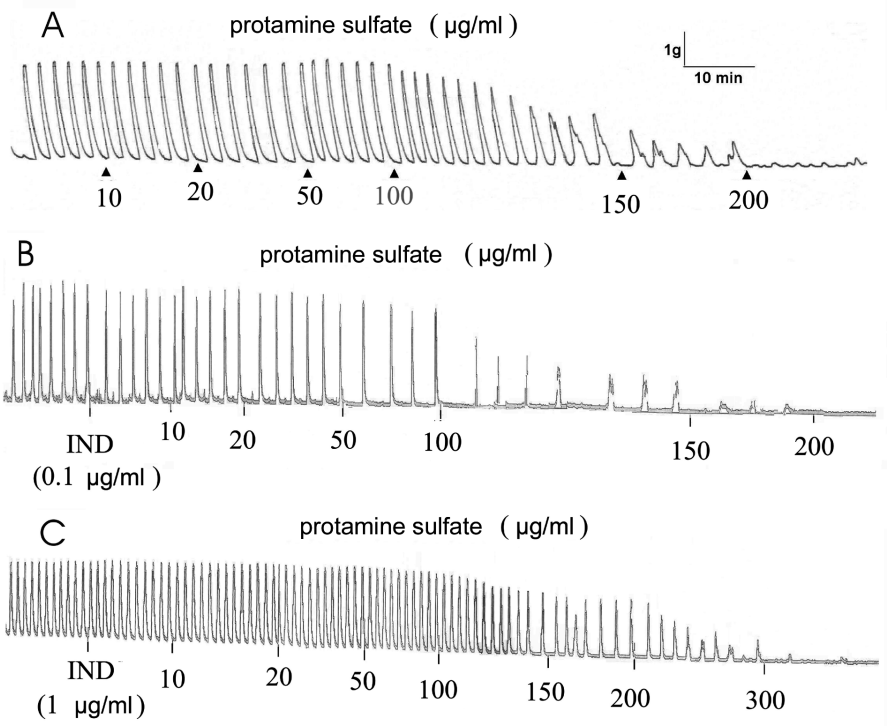

Fig. 1. Original trace (spontaneous uterine activity): (**A**) PS relaxatory effect; (**B**) Effect of indometacin (0.1 μg/ml) pretreatment on PS-induced relaxation; (**C**) Effect of indometacin (1 μg/ml) pretreatment on PS-induced relaxation (n = 7)

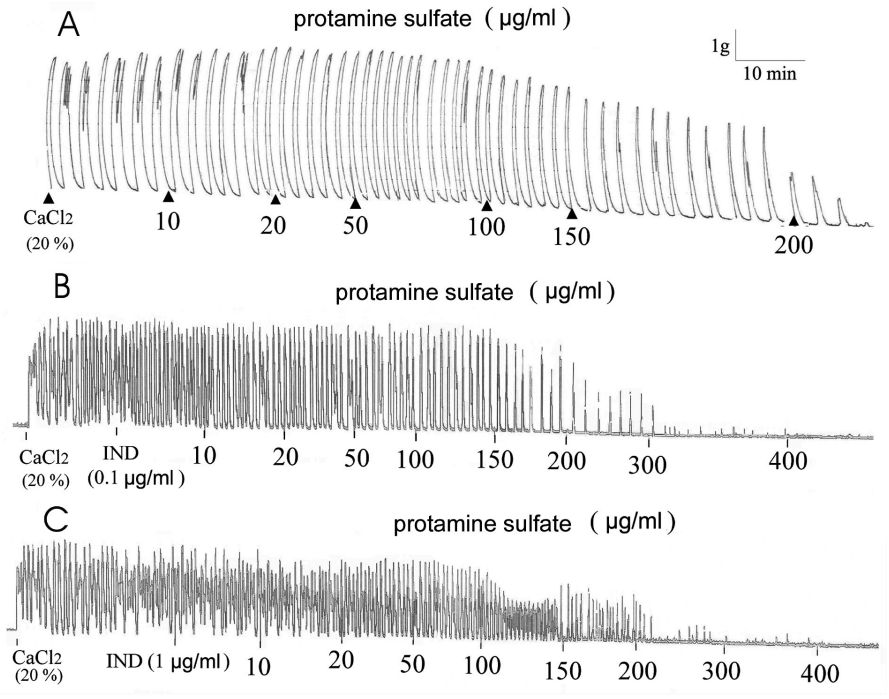

Fig. 2. Original trace (Ca^{2+}-induced uterine activity): (**A**) PS relaxatory effect; (**B**) Effect of indometacin (0.1 μg/ml) pretreatment on PS-induced relaxation; (**C**) Effect of indometacin (1 μg/ml) pretreatment on PS-induced relaxation (n = 7)

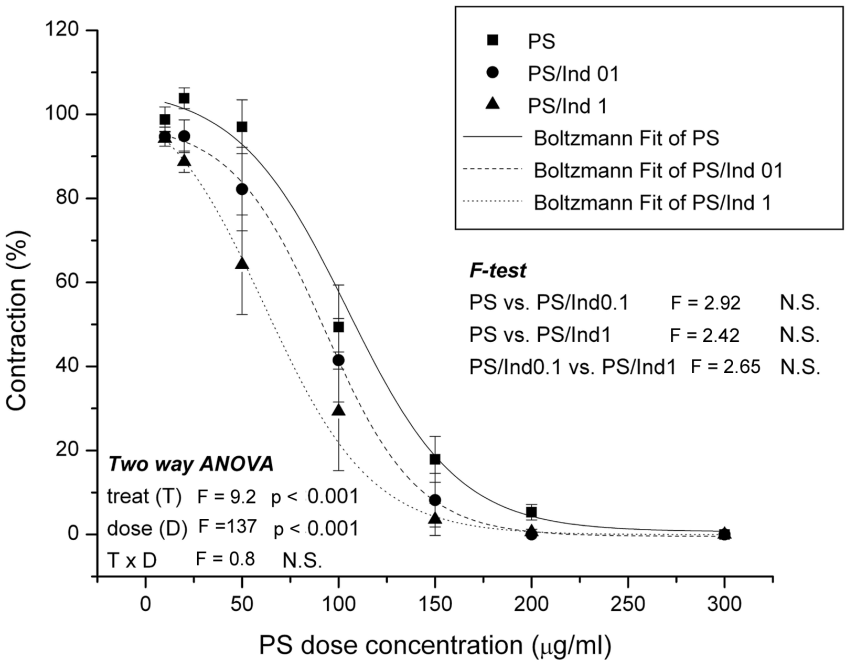

Fig. 3. Dose-response sigmoid fit curves for PS-induced relaxation of spontaneous rhythmic activity of the isolated rat uterus pretreated with indometacin (0.1 and 1 μg/ml). Data are expressed as the mean values ± SE (n = 7). The sigmoid fits were performed according to the Boltzmann equation. The results of statistical analyses using two-way ANOVA (factors: treatment (t) and dose (d)) and the F-test are given (F factors and p values)

and the higher dose was more effective (1 μg/ml) (Fig. 3). There were no differences in the shapes of the curves between the two doses investigated (non significant F values from F-test) (Fig. 3).

PS also caused dose-dependent relaxation of calcium ion-induced uterine contractions (two-way ANOVA, $p < 0.001$, $n = 7$) (Fig. 4 and Fig. 2A). Indometacin pre-treatment both decreased PS-induced relaxation of uterine contractions (two-way ANOVA, significant treatment effect, $p < 0.001$, $n = 7$) and changed the slopes of the curves of PS-induced relaxation at both concentrations of indometacin (two-way ANOVA, $p < 0.001$ and significant F-test values, PS/Ind 0.1 *vs.* PS/Ind 1, $p < 0.001$) (Fig. 4).

The EC_{50} values for PS-treated uteri were analyzed by two-way ANOVA (factors: type of contraction and treatment) followed by *post-hoc* analysis using the Newman-Keuls test for multiple comparisons (Fig. 5).

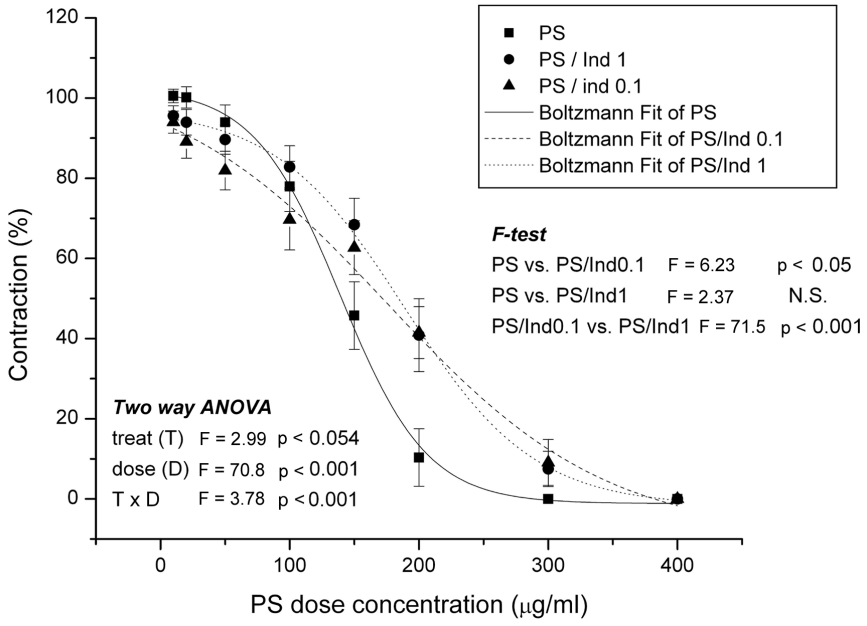

Fig. 4. Dose-response sigmoid fit curves for the PS-induced relaxation of calcium ion-induced rhythmic activity of the isolated rat uterus pretreated with indometacin (0.1 and 1 g/ml). Data are expressed as the mean values ± SE (n = 7). The sigmoid fits were performed according to the Boltzmann equation. The results of statistical analyses using two-way ANOVA (factors: treatment (t) and dose (d)) and the F-test are given (F factors and p values)

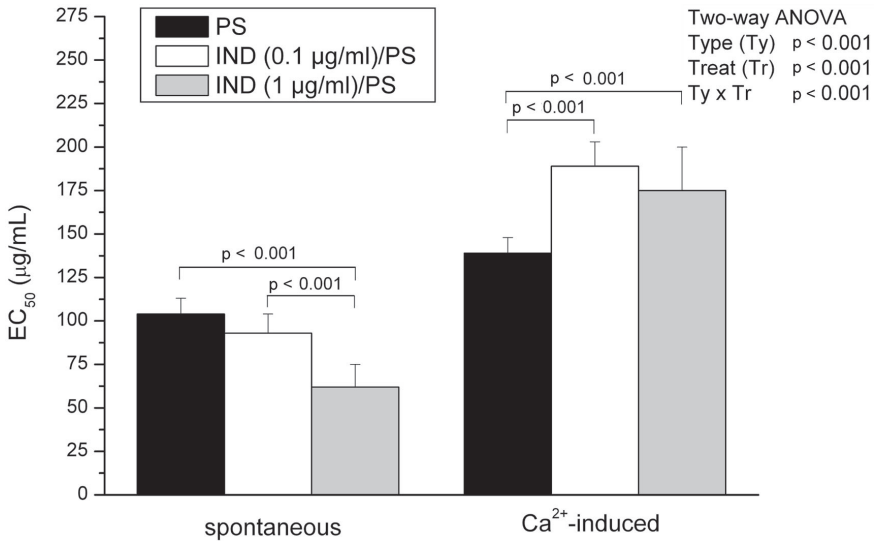

Fig. 5. The EC_{50} values for PS-treated uteri with and without pretreatment with indometacin (0.1 and 1 µg/ml). Statistical significance was tested by two-way ANOVA (factors: type of contraction and treatment) and compared *post-hoc* using Tukey's HSD test. The results indicate statistically significant differences in the results (EC_{50} values) obtained based on the type of contraction ($p < 0.001$) as well as the treatment ($p < 0.001$). The EC_{50} value was lower in spontaneously active uteri compared with those exposed to calcium ion-induced contractions. *Post-hoc* comparisons reveal that spontaneously active uteri pretreated with indometacin (1 µg/ml) have significantly lower EC_{50} values compared to the other two conditions (0.1 µg/ml indometacin pretreatment and PS effect alone). In contrast, calcium ion-induced active uteri pretreated with indometacin had significantly higher EC_{50} values

Statistically significant differences in EC_{50} values were found with regard to the type of contraction used ($p < 0.001$) and the treatment used ($p < 0.001$). The EC_{50} was lower in spontaneously active uteri than in calcium ion-induced uteri (*post-hoc* comparison, $p < 0.001$). *Post-hoc* comparison revealed that the EC_{50} values for spontaneously active uteri pretreated with indometacin (1 µg/ml) were significantly lower than the other two EC_{50} values obtained ($p < 0.001$). However, calcium ion-induced active uteri pretreated with indometacin presented higher EC_{50} values when compared to PS-treated uteri ($p < 0.001$).

Compared to non-treated controls, PS treatment did not lead to any statistically significant changes in the antioxidant enzyme levels detected at the end of experiments (Fig. 6 and 7). However, when compared to the effects of PS alone, indometacin pretreatment increased the CuZnSOD ($p < 0.001$) activities and slightly increased the GR activities ($p < 0.05$) measured after PS treatment in spontaneously active uteri (Fig. 6). In calcium ion-induced contractions, indometacin pre-treatment increased the CuZnSOD ($p < 0.001$), GSH-Px ($p < 0.001$) and GR ($p < 0.001$) activities measured after experiments with PS treatment alone (Fig. 7).

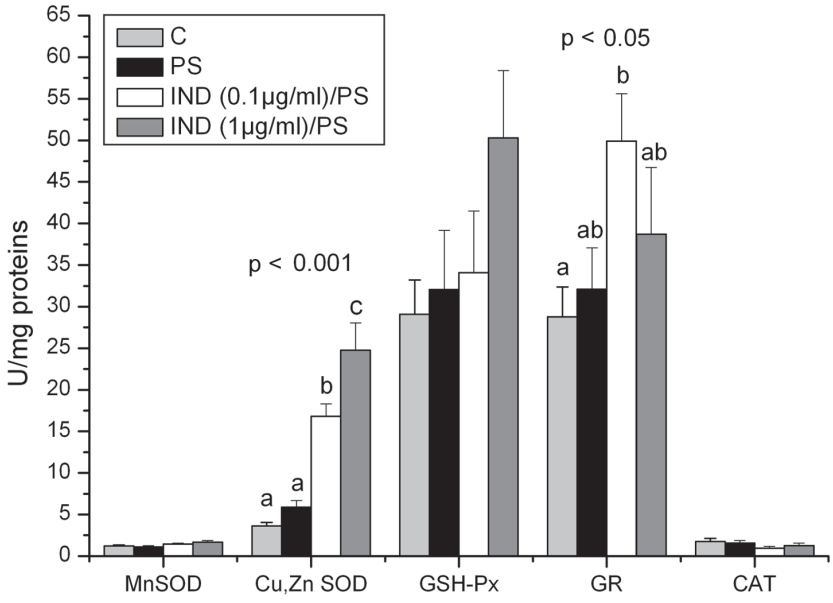

Fig. 6. Antioxidant enzyme activities in spontaneously active uteri: 1) untreated rat uteri incubated in an isolated organ chamber with De Jalon's solution for the equivalent time (2 h at 37 C, n = 8); 2) PS treated uteri (n = 7) results obtained for increasing concentrations of PS until total relaxation was achieved; 3) uteri pretreated with indometacin (0.1 µg/ml) and receiving increasing PS concentrations until total relaxation was achieved (n = 7) and 4) uteri pretreated with indometacin (1 µg/ml) followed by increasing PS concentrations until total relaxation is achieved (n = 7)

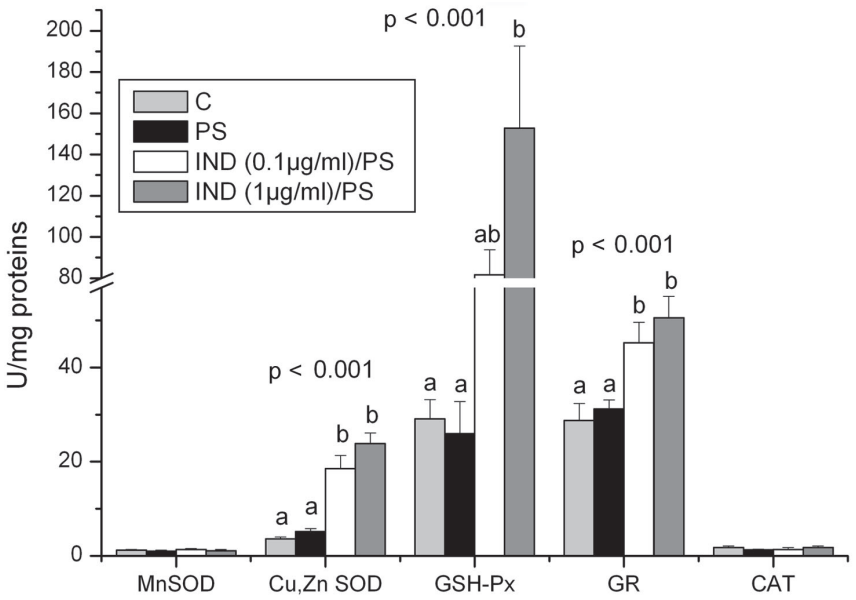

Fig. 7. Antioxidant enzyme activities in calcium ion-induced actively contracting uteri: 1) untreated rat uteri incubated in an isolated organ chamber with De Jalon's solution for the equivalent time (2 h at 37 C, n = 8) (C); 2) PS treated uteri (n = 7) in which administered concentrations of PS were increased until total relaxation was obtained; 3) uteri pretreated with indometacin (0.1 µg/ml) followed by the administration of increasing PS concentrations until total relaxation was achieved (n = 7) and 4) uteri pretreated with indometacin (1 µg/ml) followed by administration of increasing PS concentrations until total relaxation was obtained (n = 7)

Discussion

Previous results from this laboratory have shown concentration-dependent PS-mediated relaxation of isolated renal and mesenteric arteries [26, 27]. These PS-induced relaxation responses do not appear to be mediated by nitric oxide (NO) or the endothelium but appear to result from alterations in calcium ion influxes and/or calcium ion release from intracellular stores [10, 25]. The relaxatory effect of PS on the smooth muscle of isolated rat uteri has been linked, at least in part, to a NO-dependent pathway downstream of cAMP and cGMP-mediated phosphorylation [25]. Furthermore, potassium ion channels significantly contribute to PS-mediated uterine relaxation. The main channels and receptors involved in the uterine contraction/relaxation process are calcium-activated potassium channels (BK_{Ca}), β_2 adrenoceptors (AR) and long-lasting (L) type calcium ion channels [5]. Our studies also indicate that, during calcium ion-induced contractions, PS can alter both the mitochondrial and cytosolic production of H_2O_2, which can modulate the redox state of potassium ion channels [3, 25].

BK_{Ca} and two delayed rectifier potassium ion currents play significant roles in the PS-induced relaxation of spontaneous contractile activity [25]. The results portrayed herein show that indometacin pretreatment increases the relaxatory effect of PS in spontaneous uterine contractions. This is in agreement with the previously reported reduction in uterine contractions through inhibition of prostanglandin synthesis [11, 41]. Yogi et al. [39] have connected ethanol-induced vasoconstriction with ROS production and calcium ion signaling. In their experiments, transient ethanol-induced increases in calcium ion concentrations were significantly inhibited in vascular smooth muscle cell cultures pretreated with indometacin. Their findings are consistent with the results of the present study, in which pretreatment with indometacin significantly increased relaxation induced by PS.

We have found that all three types of potassium ion channels, particularly K_{ATP}, play crucial roles in the PS-induced relaxation of calcium ion-induced contractions in smooth uteri muscle [25]. Indometacin does not prevent effects that are mediated by the activation of K_{ATP} channels [4]. This indicates that the effects of indometacin pretreatment on PS relaxation of uteri involves mechanisms other than K^+-channels. Indometacin pretreatment significantly decreased PS-mediated relaxation of calcium ion-induced uterine contractions. It seems that, when compared to spontaneously contracting uteri, the external addition of calcium ions (used to induce contractions) to uteri pretreated with indometacin was sufficient to preserve internal calcium ion concentrations and slow down PS-mediated relaxation.

In calcium ion-induced active uteri, the slopes of indometacin pretreatment curves (0.1 and 1 µg/ml) were different from each other (Fig. 4). This implies the existence of different kinetics of cellular molecu-

lar events when cells were treated with different doses of indometacin. Indometacin is strongly associated with lipid membranes, which influence the localization, structure and function of membrane-associated proteins and regulate events during cellular signaling. Thus, it is possible that, independently of its cyclooxygenase inhibitory effects, indometacin regulates diverse cell functions by altering micro-environments within the membrane. It may be that the effects of indometacin reported herein are due to indometacin-induced changes in the phase behavior of membrane lipids [40]. In this regard, it is noteworthy that the functionality of BK channels depends on their compartmentalization into lipid raft microdomains [31], as the disruption of rafts has a profound effect on uterine contractility and calcium ion signaling [33].

In our experiments, indometacin pretreatment elevated CuZnSOD, indicating greater dismutation of superoxide and H_2O_2 production in the cytosol. In addition, GR activity slightly increased in spontaneously active uteri after indometacin pretreatment. In calcium ion-induced contractions, indometacin pretreatment also increased GSH-Px and GR activities, indicating greater utilization and turnover of glutathione. These results indicate that the ADS system acts by opposing a state of disrupted redox homeostasis, trying to prevent damage *via* oxidative stress and to preserve the cellular redox state. It is possible that calcium ion release channels in the sarcoplasmic reticulum increase their activities *via* the oxidation of accessible protein thiols, an effect that may be reversed following reduction with dithiothreitol (DTT) [1]. Furthermore, sarcoplasmic reticulum-mediated calcium ion reuptake is inhibited by high concentrations of H_2O_2, and the effect is reversed by DTT [2]. Finally, oxidants alter myofibrillar calcium ion sensitivity both in time- and concentration-dependent fashions indicating that a precise balance of ROS concentrations in contractile cells is necessary. Indeed, H_2O_2 has been shown to decrease calcium ion binding to calmodulin [38]. The relaxatory effect of H_2O_2 on smooth muscles has been confirmed in isolated uterine muscle [3]. The role of lipid peroxides in this process is still unresolved. Furthermore, the indometacin doses applied in our experiments have been found to elevate oxygen uptake, uncouple oxidative phosphorylation and decrease cellular ATP levels [14], suggesting a state of disturbed redox equilibrium and increased ROS turnover.

Our results provide evidence that, in addition to its influence on prostaglandin synthesis and cellular calcium ion homeostasis, indometacin also influences the effects of PS by strongly associating with lipid membranes, thereby influencing the localization, structure and function of membrane-associating proteins and actively regulating cell signaling events. Therefore, it is possible that, in addition to its action as a cyclooxygenase inhibitor, indometacin regulates diverse cell functions by altering micro-environments within cellular membranes. Under the experimental conditions employed in this study, the extent of these effects differed between spontaneous and calcium ion-induced contracting uteri. The processes appear to be mediated by redox reactions involving energy metabolism, ROS, lipid peroxides and AD enzymes, the complexities of which remain to be fully elucidated.

Acknowledgment:
This work was supported by a grant from the Ministry of Science of the Republic of Serbia, project No. 143034B University of Belgrad, "The role of redox-active substances in the maintenance of homeostasis".

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Received: August 18, 2010; **in the revised form:** February 24, 2011; **accepted:** March 3, 2011.