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Research article

Diethyldithiocarbamate potentiates the effects of protamine sulphate in the isolated rat uterus

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Protamine sulphate causes potassium ion channel-mediated relaxation of spontaneous and calcium ion-induced contractions of the isolated rat uterus. Diethyldithiocarbamate (DDC) potentiated the effect of protamine sulphate. A mechanism for DDC's action was postulated on the basis of its interactions with divalent iron ions and Cu,Zn-SOD. DDC chelates divalent iron ions creating DDC-iron (Fe-DDC) complexes. Fe-DDC forms stable NO-Fe-DDC_2 complexes by NO scavenging and de-nitrosylation processes, which in combination with DDC (5 mM) provoke inhibition of Cu,Zn-SOD resulting in specific oxidative conditions culminating in potassium ion channel opening, membrane hyperpolarisation, inhibition of calcium ion influx and subsequent muscle relaxation. As Fe-DDC and NO-Fe-DDC_2 complexes exclude divalent iron ions from participating in the hydroxyl radical generating Fenton reaction, DDC can also prevent iron-related pathophysiological manifestations. Such permissive roles of DDC open the possibility for application of its pharmacological form (disulfiram) to a wider spectrum of pathophysiological conditions related to smooth muscles.

Keywords: protamine sulphate, diethyldithiocarbamate, uterus, nitric oxide

Introduction

Protamine sulphate has multiple physiological effects mediated via different mechanisms. Some studies have suggested that the cardiovascular depressant actions of protamine sulphate result from a direct effect producing aberrant conduction within the heart that may result in deleterious effects on heart function typical of myocardial disease.[1] The main mechanism of action of protamine sulphate in smooth muscle is via interactions with calcium influxes and/or calcium ion release from intracellular stores.[2] The main role of calcium ions in contraction of uterine smooth muscle is the activation of calcium ion calmodulin-dependent protein kinase and phosphorylation of myosin, a process dependent on ATP consumption. In response to ATP depletion, K_{ATP} channels open leading to potassium ion efflux and membrane hyperpolarisation. This lowers the electrical activity of the cell and energy consumption thereby linking metabolic state to excitability.[3] In addition to

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Abbreviations: Cu,Zn-SOD, copper-zinc superoxide dismutase; DDC, diethyldithiocarbamate; DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide; EPR, electron paramagnetic resonance; ROS, reactive oxygen species, RSNO, S-nitrosothiols

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ATP depletion, reactive oxygen species (ROS) operate as physiological activators of K_{ATP} channels.[4] Various steps of the contractile process have proven to be susceptible to redox modulation. First, the probability of isolated sarcoplasmic reticulum (SR) calcium ion release channels opening increases upon oxidation of accessible protein thiols. Second, SR calcium ion re-uptake is inhibited by high concentrations of H_2O_2 and third, oxidants alter myofibrillar calcium ion sensitivity in both a time- and concentration-dependent fashion.[5]

Diethyldithiocarbamate (DDC) is known to be a potent copper ion chelating agent and is widely used as a copper-zinc superoxide dismutase (Cu,Zn-SOD) inhibitor both *in vivo* and *in vitro*.[6–8] The mechanism of DDC-mediated Cu,Zn-SOD inhibition has been well described. Two molecules of DDC are required; one extracts the copper ion from the active site while the second molecule replaces the copper ion in the active site resulting in enzyme inhibition. The degree of inhibition is dose dependent from very low up to almost 100%.[7] The effects of DDC on biological systems may also result from S-nitrosothiol (RSNO) elimination.[9] A third mechanism of action was revealed when the effect of DDC on the vaso-relaxatory activity of acetylcholine, nitric oxide (NO), nitrite, glyceryl trinitrate and dinitrosyl iron cysteine complexes was studied in isolated rat aortic rings contracted with norepinephrine. Pretreatment of the rings with DDC attenuated the vasodilator-induced vasorelaxation and prevented the sub-sequent restoration of vessel tone. The inhibitory effects of DDC occur due to the trapping of NO by the divalent iron–DDC complex which is formed in the tissue.[10] Contrary to common belief, NO radicals are trapped in biosystems by iron-diethylthio-carbamates not only in the ferrous, but also in the ferric, state. In addition, the NO moiety can bind to some other molecule that can be donated to iron–DDC complexes in a transnitrosation reaction. This has been shown for dinitrosyl-iron complexes (DNICs) that endogenously form in living tissues. The balance between free/bound iron and different iron ligands seems to have an important physio-logical role by affecting the metabolism of reactive nitrogen and oxygen species.[11]

Mechanisms underlying DDC's action remain complex and not fully understood. In this current study, we have investigated the effects of two different concentrations of DDC applied to isolated virgin rat uterine muscle *in vitro* exhibiting protamine sulphate-induced inhibition of both spontaneous and calcium ion-induced rhythmic contractions.

Materials and methods

Tissue preparation and contractility recording

Isolated uteri from virgin Wistar rats (body weight 200–250 g) in the oestrous phase of the oestrous cycle, determined by vaginal smear cytology,[12] were used in this study. Uteri were suspended in an isolated organ bath chamber (Experimetria, Budapest, Hungary) containing De Jalon's solution (in g/l: NaCl 9.0, KCl 0.42, $NaHCO_3$ 0.5, $CaCl_2$ 0.06, glucose 0.5) and aerated with 95% O_2 and 5% CO_2. The temperature was maintained at 37°C. All protocols for handling the rats were approved by the local ethical committee for animal experimentation that strictly follows inter-national regulations. Isometric contractions were recorded using an isometric force transducer (Experimetria). The preload of the preparation was approximately 1 g.

Experimental procedures

One uterus (control C1) was immediately frozen using liquid nitrogen before being transferred to –80°C until analysis. The other (control C2) was suspended in an isolated organ bath containing De Jalon's solution and, after 2-h equilibration, was frozen using liquid nitrogen. These uteri served as controls for the determination of antioxidative enzyme activities. After 30 min of establishing stable spontaneous and calcium ion-induced contractions (by the addition of 20% $CaCl_2$ solution into the isolated organ bath to adjust the final concentration to 180 mM), the uteri were incubated with increasing concentrations of protamine sulphate until total cessation of contractions took place. In order to test the involvement of redox mechanisms in the mechanism of action of protamine sulphate, two different concentrations of DDC were used (2.5 mM and 5 mM). Each concentration of DDC was added to the De Jalon's solution 10 min before protamine sulphate addition. Protamine sulphate-induced relaxation was expressed as the ratio of maximal tension observed in the presence of DDC (relative ratio). All uteri were frozen using liquid nitrogen at the end of the experiment.

For the determination of SOD activity, the thawed uteri were homogenised and sonicated in ice-cold 0.25 M sucrose, 1 mM EDTA and 0.05 M Tris-HCl buffer (pH 7.4). SOD was assayed using the method of Misra and Fridovich.[13] SOD activity was expressed in units per milligram of protein.

The effects of DDC on SOD activity were also tested before pharmacological experimental procedures. Isolated, untreated, homogenised and sonicated uteri

were used as the control (C). Other homogenates were treated with DDC (2.5 mM and 5 mM).

Fenton reaction

The Fenton reaction was performed by combining 6 mM H_2O_2 and 2 mM $FeSO_4$ in 200 mM phosphate buffered solution (pH 7.4), made by combining solutions of NaH_2PO_4 and Na_2HPO_4 in ultra-pure (18.2 MΩ milliQ) water. EPR spin-trap DEPMPO (Alexis Biochemical, Lausen, Switzerland) was added at a final concentration of 28 mM, prior to the addition of H_2O_2. In experiments employing DDC, a final concentration of 2.5 mM was used (prior to the addition of H_2O_2). EPR spectra were recorded at room temperature using a Varian E104-A ESR spectrometer operating at X-band (9.572 GHz), using the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW; field centre, 3410 G. Factor g was calculated using the equation: $g = h\nu/\beta_e B$. All spectra were recorded using EW software (Scientific Software, Bloomington, IL, USA). Measurements were performed using quartz capillaries in which Teflon tubes with samples were placed. All experiments were performed in triplicate.

Drugs and solutions

Protamine sulphate was supplied by Galenika a.d. (Belgrade, Serbia). DDC was purchased from Sigma-Aldrich (St Louis, MO, USA); H_2O_2 was from Renal (Budapest, Hungary) and $FeSO_4$ was from Merck (Darmstadt, Germany). 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).

Statistical analysis

Statistical analyses (descriptive statistics and analysis of variance, ANOVA) were performed according to protocols described by Hinkle *et al.*[14] and Manley[15]

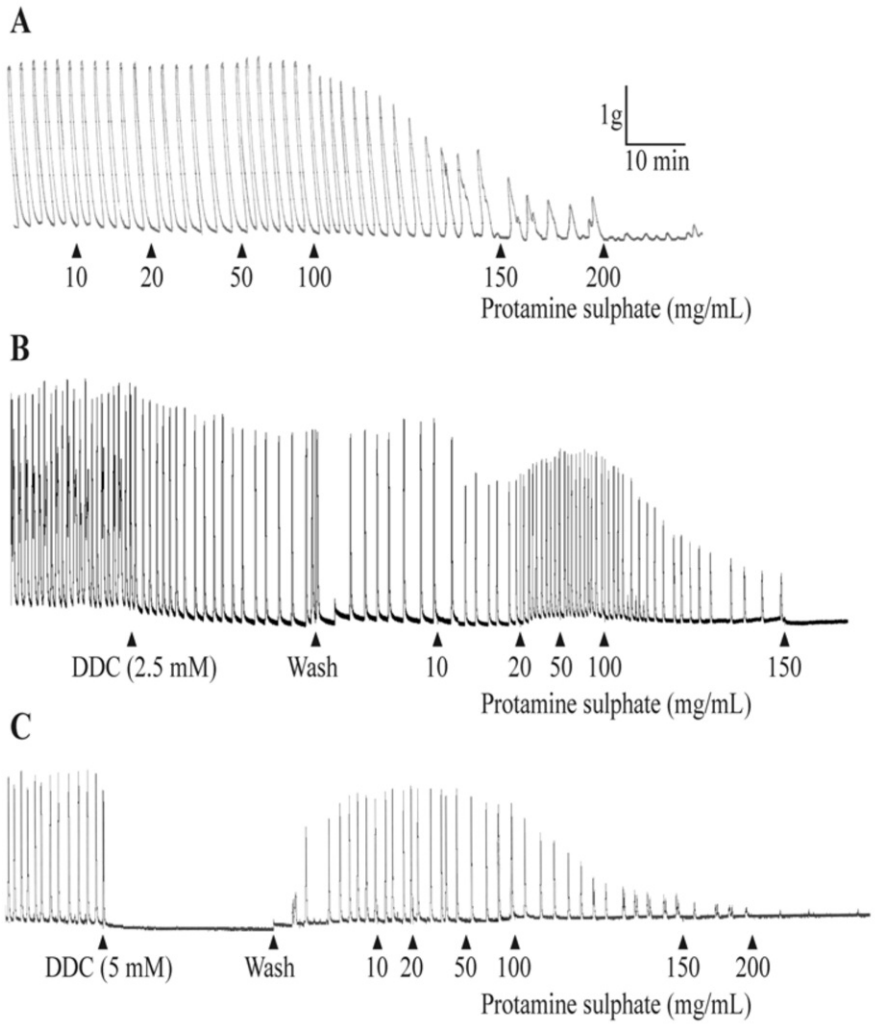


Figure 1 Representative recordings of protamine sulphate-mediated relaxation of spontaneous rhythmic activity of the isolated rat uterus. (A) Treated with protamine sulphate. (B) Pretreated with DDC (2.5 mM), washed and then treated with protamine sulphate. (C) Pretreated with DDC (5 mM), washed and then treated with protamine sulphate

using Statistical Analyzer Set (SAS v.9.1.3; SAS Institute Inc, Cary, NC, USA). All data are expressed as the mean ± SEM. Differences between groups were tested by two-way ANOVA with treatment and dose as factors and were considered statistically significant when $P < 0.05$. The curves were *post hoc* compared using Duncan's range test and regression analysis of linear fitted dose-response curves.

Results

Protamine sulphate caused dose-dependent relaxation of spontaneous contractile activity of the isolated rat uterus (Fig. 1A). The presence of 2.5 mM DDC induced moderate relaxation of spontaneous contractile activity. After removing DDC by washing, the relaxatory effects of protamine sulphate on spontaneous uterine contractions were increased (Fig. 1B). The presence of 5 mM DDC caused complete cessation of contractile activity. Removal of DDC by washing led to reoccurrence of spontaneous contractions (Fig. 1C). Pre-treatment with 5 mM DDC caused a greater effect on protamine sulphate-mediated relaxation of spontaneous contractile activity in comparison with 2.5 mM DDC. Similar results were obtained when uterine contractions were calcium ion-induced (Fig. 2A–C). Figure 3 shows dose-response curves for protamine sulphate-induced relaxation of spontaneous and calcium ion-induced rhythmic activity of the isolated uterus untreated or pretreated with DDC.

We measured Cu,Zn-SOD and Mn-SOD activity in all uterine preparations: in control uteri, 2.5 mM DDC treated uteri, 5 mM DDC treated uteri, protamine sulphate treated uteri (spontaneous and calcium ion-induced), protamine sulphate treated uteri pretreated with 2.5 mM DDC (spontaneous and calcium ion-induced) and protamine sulphate treated uteri pretreated with 5 mM DDC (spontaneous and

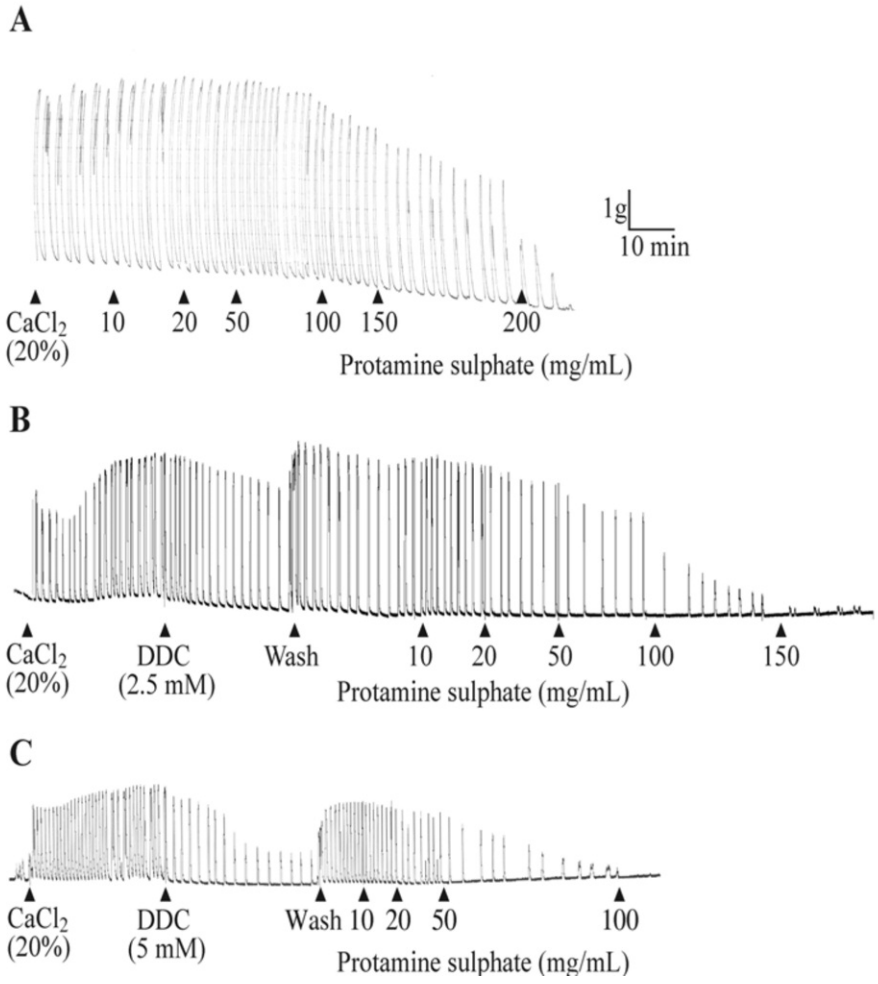


Figure 2 Representative recordings of protamine sulphate-mediated relaxation of calcium ion-induced rhythmic activity of the isolated rat uterus. (A) Treated with protamine sulphate. (B) Pretreated with DDC (2.5 mM), washed and then treated with protamine sulphate. (C) Pretreated with DDC (5 mM), washed away and then treated with protamine sulphate

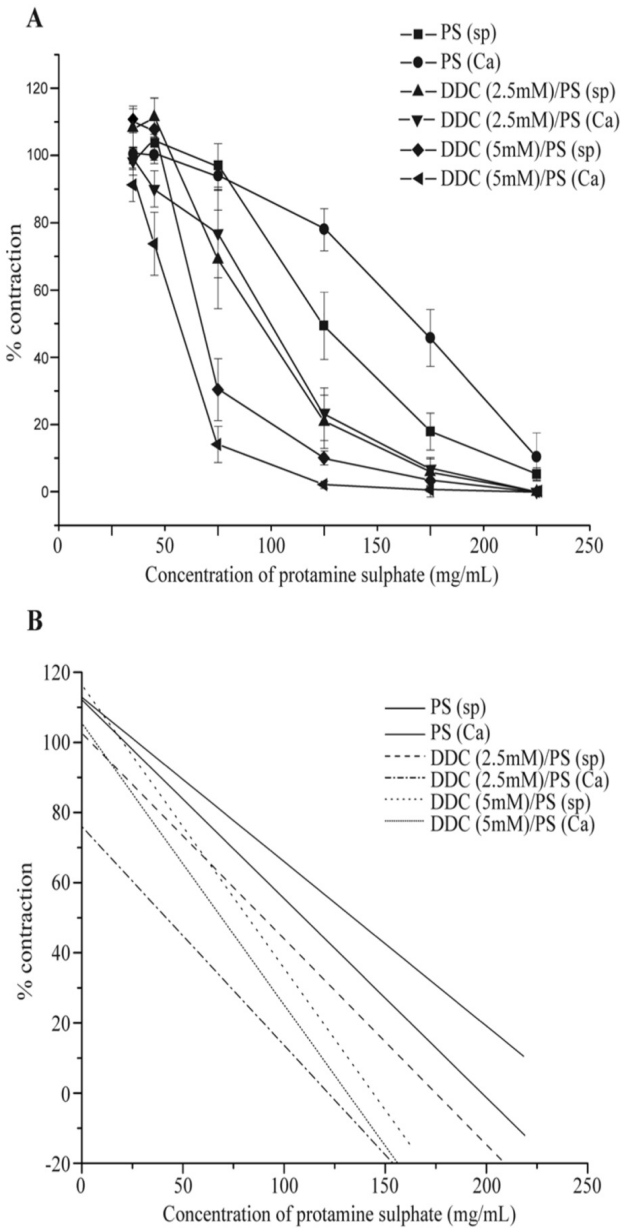


Figure 3 (A) Dose-response curves for protamine sulphate-induced relaxation of spontaneous and calcium ion-induced rhythmic activity of the isolated rat uterus pretreated with DDC (2.5 mM) and DDC (5 mM). Data are expressed as mean ± SEM (n = 7). (B) Regression analysis of protamine sulphate-induced relaxation of spontaneous and calcium ion-induced rhythmic activity of the isolated rat uterus pretreated with DDC (2.5 mM) and DDC (5 mM). Regression lines are presented (n = 7, for each concentration and pretreatment, R-coefficient of regression)

calcium ion-induced). Only 5 mM DDC treatment resulted in significant inhibition of endogenous Cu,Zn-SOD (Table 1). In contrast, an increase in Mn-SOD activity was observed (Table 1).

Table 1 Cu,Zn-SOD and Mn-SOD enzyme activities in isolated rat uteri

Enzyme	Control	DDC (2.5 mM)	DDC (5 mM)
Cu,Zn-SOD	3.20 ± 0.30	4.30 ± 1.20	1.30 ± 0.60*
Mn-SOD	0.71 ± 0.14	0.83 ± 0.05	1.25 ± 0.15*

Values expressed in U/mg of protein.
Control (incubated in De Jalon's solution for 2 h and then frozen in liquid nitrogen), 2.5 mM DDC treated and 5 mM DDC treated uteri were used. One-way ANOVA was performed to analyze differences between experimental groups.
*Statistically significant when compared to controls ($P < 0.05$).

DDC inhibited hydroxyl radical generation in the Fenton reaction *in vitro* (Fig. 4), thereby stabilising H_2O_2. On the basis of our results, we have postulated a mechanism for DDC's action (Scheme 1).

Discussion

These results have unequivocally demonstrated that pretreatment with DDC enhances relaxation provoked by protamine sulphate. The addition of DDC to myocyte cell cultures induces dose-dependent inhibition of SOD (1 mmmM DDC causes 33% inhibition and 1 mM causes 63% inhibition) after 72 h.[17] DDC has been shown to inhibit erythrocyte Cu,Zn-SOD activity in healthy subjects, in familial amyotrophic lateral sclerosis (Leu144Phe) patients and in sporadic amyotrophic lateral sclerosis patients. However, its inhibitory effect was more pronounced in familial amyotrophic lateral sclerosis patients.[18]

In the present study, pretreatment with 5 mM DDC resulted in endogenous Cu,Zn-SOD inhibition while

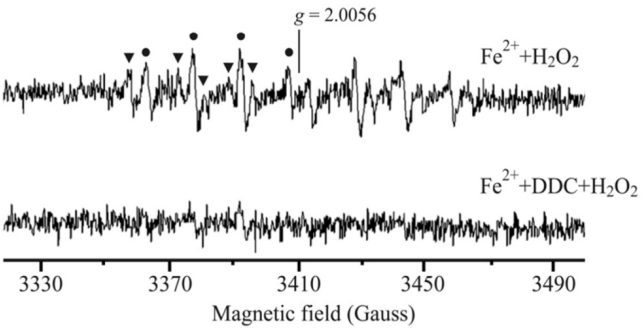
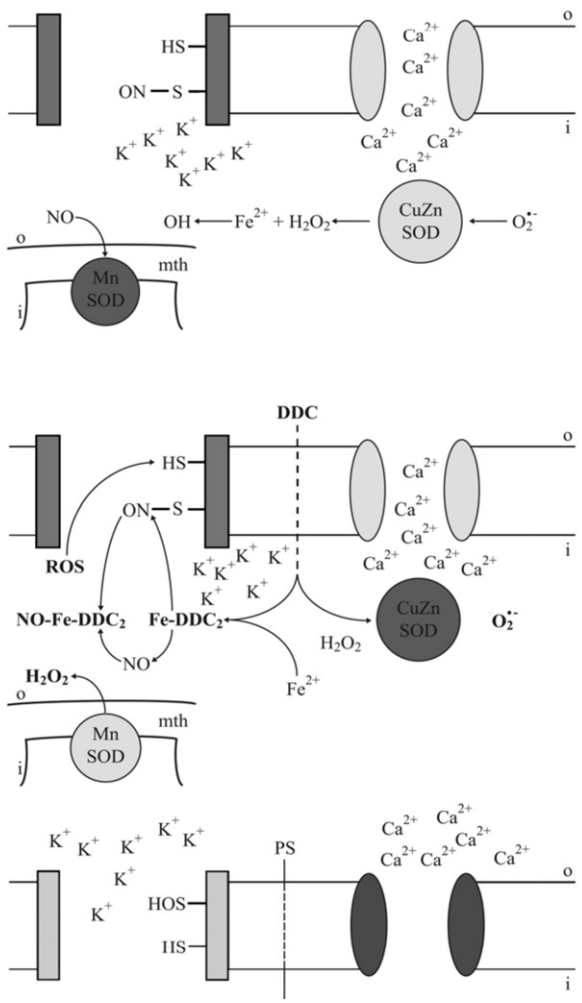


Figure 4 Characteristic EPR spectra of DEPMPO adducts formed in the Fenton system at pH 7.4 (top) and in the Fenton system with the addition of DDC (2.5 mM) (bottom). Filled circles, characteristic lines of the DEPMPO/OH adduct; inverted triangles, characteristic lines of the DEPMPO/H adduct, a species previously proposed to be generated in the Fenton reaction at physiological pH.[16] The centre of signals of DEPMPO/OH and DEPMPO/H are at g = 2.0056, as illustrated.

2.5 mM DDC was without effect. Such a difference did not affect DDC's potentiating effect on protamine sulphate-induced relaxation indicating that the main mechanism of DDC's effect on protamine sulphate-induced relaxation was not due to SOD inhibition. We recently demonstrated that K_{ATP} channels play a crucial role in protamine sulphate-provoked relaxation of smooth muscles, particularly when contractions are calcium ion-induced.[19] In fact, we postulated an alternative explanation for DDC's effect. Potassium ion channels can be modulated by both pro-oxidants and by nitrosylation. DDC is known to lead to the formation nitric oxide–iron–DDC (NO-Fe-DDC_2) complexes which can modulate the intracellular environment in such a way as to affect potassium ion channels. Fe–DDC complexes may readily bind NO radicals to form stable mononitrosyl and dinitrosyl iron complexes *in vivo*. Fe–DDC complexes may scavenge free NO and/or carry out denitrosylation reactions. Therefore, in muscle cells, Fe–DDC complexes could denitrosylate thiol groups within potassium ion channels leading to potassium ion efflux and membrane hyperpolarisation, particularly when oxidized by ROS (Scheme 1). The ability of these molecules to bind NO could determine the levels of intracellular NO or NOderived species such as peroxynitrite in diethyldithiocarbamate-based therapy (*e.g.* disulfiram). Fe–DDC complexes have undisputed merits as scavengers of NO.



Scheme 1 Mechanism of DDC-induced relaxation. Top: K^+ channels are blocked by nitrosylation of cysteine residues resulting in calcium ions (Ca^{2+}) entering the cell which leads to muscle contractions. Cu,Zn-SOD removes superoxide ($O_2^{\bullet-}$) that generates H_2O_2 which participates with divalent iron ions in the Fenton reaction. Centre: DDC enters the cell and chelates divalent iron ions. DDC–divalent iron ion (Fe–DDC) complexes remove NO into the cytosol creating NO–Fe–DDC_2. DDC also inhibits Cu,Zn-SOD leading to an increased level of $O_2^{\bullet-}$. Under such pro-oxidative conditions, ROS ($O_2^{\bullet-}$ and H_2O_2) oxidize cysteine residues in K^+ channels which leads to channel opening. Bottom: efflux of K^+ through the open channels leads to hyperpolarisation of the membrane preventing Ca^{2+} influx. Therefore, muscle relaxation and decreased excitability ensues which amplifies the effects of protamine sulphate. Light grey, active channel or enzyme; dark grey, inactive channel or enzyme.

Inhibition of Cu,Zn-SOD activity by pretreatment with 5 mM DDC in combination with an increase in Mn-SOD activity indicates that increased ROS production (increased superoxide in cytosol and H_2O_2 production in mitochondria) may play an additional role in DDC's effect. While previous studies have indicated that H_2O_2 causes both contraction and relaxation[20–22] and, in some cases, dose-dependent biphasic response,[23,24] the latest results show that relaxation represents the prevailing effect.[25] As ROS target protein thiol groups,[26] it has been suggested that smooth muscle K_V-channels are activated by oxidation causing smooth muscle relaxation. Inhibition of intracellular signalling provoked by pro-oxidative conditions related to DDC therapy could be prevented by DDC's ability to chelate divalent iron ions thereby preventing the hydroxyl radical generating Fenton reaction that represents a serious pathophysiological hazard (Fig. 4 and Scheme 1).

The recognition of pleiotropic multisite actions of Fe–DDC complexes is reinforced by our current results in that the permissive role of endogenously formed Fe–DDC complexes increase the capacity of pharmacological forms of DDC such as disulfiram to be used in the treatment of a wide spectrum of diseases.[27,28]

Acknowledgement

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