

# Diethylthiocarbamate inhibits the catalytic activity of xanthine oxidase

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**Abstract** We sought to determine the effects of the superoxide dismutase (SOD) inhibitor diethylthiocarbamate (DETC) on vascular superoxide production. Rat aortic rings treated with DETC (10 mM) showed no change of superoxide generation (5  $\mu$ M lucigenin). Likewise, DETC did not change the expression and activity of vascular soluble guanylyl cyclase, an enzyme known to be extremely sensitive to superoxide. In striking contrast, DETC completely inhibited the superoxide production induced by 6-anilino-5,8-quinolinedione (LY83583) and abolished the catalytic activity of xanthine oxidase (XO). Thus, DETC inhibits vascular superoxide production by blocking oxidoreductase enzymes such as XO and those reducing LY83583 in rat aorta.

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**Key words:** Diethylthiocarbamate; 6-Anilino-5,8-quinolinedione; Superoxide; Xanthine oxidase; Soluble guanylyl cyclase

## 1. Introduction

Vascular oxidative stress has been shown to contribute to a variety of cardiovascular diseases such as coronary artery disease, heart failure, diabetes and hypertension. The mechanism underlying increased bioavailability of vascular reactive oxygen species is multifactorial and involves increased expression and activity of enzymes generating oxygen radicals such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a decreased expression and activity of enzymes which detoxify reactive oxygen species such as superoxide dismutases (SODs) and a decreased concentration of typical cellular antioxidants such as cysteine and glutathione [1,2]. These mechanisms are currently investigated in many research laboratories by the use of low molecular activators and inhibitors of enzymes which either produce or detoxify reactive oxygen species.

Diethylthiocarbamate (DETC) is a metal ion-chelating agent which is a well-known inhibitor of SODs [3]. In addition, DETC has been described as an inhibitor of nuclear

factor (NF)  $\kappa$ B and it was found that DETC is able to trap nitric oxide (NO) [4,5]. These activities should result in an increase of vascular superoxide production in tissues including the vasculature. Indeed, the results of previous investigations in atherosclerotic rabbit aorta indicate that this seems to be the case [6,7]. In an attempt to investigate the effects of DETC on vascular superoxide production stimulated with 6-anilino-5,8-quinolinedione (LY83583), a quinone derivative known to generate superoxide intracellularly [8], we did not find a further increase but an almost complete inhibition of the stimulated superoxide generation. Thus, we sought to determine if DETC significantly impacts on oxidoreductases in the vasculature.

## 2. Materials and methods

### 2.1. Preparation and incubation of isolated thoracic aorta

We investigated isolated thoracic aortic rings of 21 normal male Wistar rats (WIS) at an age of 3–4 months. Aortas were excised after removal of the hearts and rapidly immersed in Krebs–HEPES buffer (pH 7.4) of the following composition (in g/l): NaCl, 5.782; KCl, 0.350; CaCl<sub>2</sub>, 0.275; MgSO<sub>4</sub>, 0.296; NaHCO<sub>3</sub>, 2.1; K<sub>2</sub>HPO<sub>4</sub>, 0.14; Na–HEPES, 5.206; D-glucose, 2.0. Two segments of each aorta were used. Segments were subjected to vehicle, LY83583 (10  $\mu$ M) and DETC (10 mM) for 4 h in Krebs–HEPES buffer in a water jacketed organ bath (37°C). The drugs were renewed every 30 min. The segments were subsequently frozen in liquid nitrogen.

### 2.2. Preparation of 100 000 $\times$ g supernatants

Frozen segments were homogenized in 2 ml Tris buffer (5 mmol/l) supplemented with dithiothreitol (DTT) (5 mmol/l) and the proteinase inhibitors leupeptin, benzamide, aprotinin, phenylmethylsulfonyl fluoride (PMSF) and antipain (10  $\mu$ g/ml). The homogenate was centrifuged at 4°C at 100  $\times$ g (10 min), 10 000  $\times$ g (15 min), and 100 000  $\times$ g (1 h). The obtained cytosolic fraction was stored in aliquots at –80°C and used for enzyme activity assays and Western blotting. Protein content was measured by the method of Bradford [9] using bovine serum albumin (BSA) as a standard.

### 2.3. Western blotting

Western blotting was performed as described previously [10]. Briefly, each lane was loaded with 10  $\mu$ g total protein, and blots were incubated with a soluble guanylyl cyclase (sGC) antibody staining the b<sub>1</sub> subunit of sGC (sGC-b<sub>1</sub>, Alexis). After incubation with a peroxidase A-conjugated antirabbit IgG (Calbiochem, Darmstadt, Germany), blots were developed using enhanced chemiluminescence (ECL) (Roche) and exposed to X-ray films.

### 2.4. Determination of sGC activity

Specific activity of sGC was measured by the formation of [<sup>32</sup>P]-cyclic guanosine monophosphate (cGMP) from [ $\alpha$ -<sup>32</sup>P]guanosine triphosphate (GTP), as described previously [11]. Briefly, sGC of aortic cytosols (20–40  $\mu$ g of protein) was incubated in a total volume of 100  $\mu$ l of a triethanolamine–HCl buffer (50 mM, pH 7.4, 37°C) containing [ $\alpha$ -<sup>32</sup>P]GTP (5 nM, 0.4  $\mu$ Ci), GTP (100  $\mu$ mol/l), cGMP (1 mmol/l), 3-isobutyl-1-methylxanthine (IBMX, 1 mmol/l), MgCl<sub>2</sub>

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**Abbreviations:** DETC, diethylthiocarbamate; DMSO, dimethylsulfoxide; DTT, dithiothreitol; LY83583, 6-anilino-5,8-quinolinedione; sGC, soluble guanylyl cyclase; SNAP, S-nitroso-N-acetylpenicillamine; SOD, superoxide dismutase; XO, xanthine oxidase; X/XO, xanthine/xanthine oxidase

(1 mmol/l) and DTT (1 mmol/l) in the presence of racemic *S*-nitroso-*N*-acetylpenicillamine (SNAP) (1  $\mu$ mol to 1 mmol/l) or vehicle (maximal 0.25% dimethylsulfoxide (DMSO)).

### 2.5. Quantification of superoxide production

Generation of superoxide radicals by thoracic aorta of the rat was measured by use of the lucigenin assay as described previously [12]. Briefly, aortic rings (5 mm width) were equilibrated in Krebs–HEPES buffer (pH 7.4, 37°C) with vehicle, LY83583 (10  $\mu$ M) or DETC (10 mM) for 30 min and then transferred to vials containing albumin buffer enriched with 5  $\mu$ M lucigenin (pH 7.4, 37°C). Superoxide production in the vessel segments was detected by measuring the luminometer counts cumulatively over 20 min. These data were corrected for background counts. Generation of superoxide was achieved by adding xanthine oxidase (XO) (1 mU/ml) to increasing concentrations of xanthine (0.1–2.4  $\mu$ M) dissolved in albumin buffer [12] with vehicle or DETC (10 mM). In another series of experiments the superoxide-induced oxidation of adrenaline to adrenochrome was monitored as described previously [13]. Briefly, 25 mU/ml XO and 50  $\mu$ M xanthine or 10–20  $\mu$ l of a saturated  $\text{KO}_2$  solution in dry DMSO were added to 2 mM adrenaline dissolved in 50 mM phosphate buffer (pH 7.6, 25°C) and formation of adrenochrome was monitored at 480 nm in the presence of 1  $\mu$ M to 10 mM DETC, 10  $\mu$ g/ml SOD, 10  $\mu$ g/ml SOD+1 mM DETC or vehicle.

### 2.6. Substances and solutions

Racemic SNAP was synthesized as described previously [14]. The stock solution of SNAP (10 mM) was prepared daily in DMSO (5%), kept on ice and protected from daylight. Grade I XO from buttermilk and bovine erythrocyte SOD were obtained from Sigma. All other chemicals were obtained from Merck, Darmstadt, Germany or from Sigma, Deisenhofen, Germany, in analytical grade. All concentrations indicated in the text, figures and tables are expressed as final bath concentrations.

### 2.7. Statistics

All data were analyzed by standard computer programs (GraphPad Prism PC Software version 3.0, analysis of variance (ANOVA)) and are expressed as mean values and standard error of the mean (S.E.M.). Significant differences were evaluated using either Newman–Keuls multiple comparison test (ANOVA) or two-way ANOVA.  $P < 0.05$  was considered significant.

## 3. Results

### 3.1. Effect of DETC and LY83583 on aortic superoxide generation

Surprisingly, the SOD inhibitor DETC had no effect on aortic superoxide generation (Fig. 1A), while LY83583 strongly increased the lucigenin chemiluminescence signals (Fig. 1B). After 20 min the rate of superoxide generation was increased from  $78 \pm 3.8$  counts/mg (vehicle) to  $147 \pm 18.4$  counts/mg (LY83583,  $P = 0.0043$ ). This increase of superoxide generation was completely inhibited by DETC (Fig. 1C). The superoxide generation measured in the presence of LY83583 and DETC ( $59 \pm 14.6$  counts/mg) was identical to control values ( $65 \pm 12.2$  counts/mg,  $P > 0.05$ ).

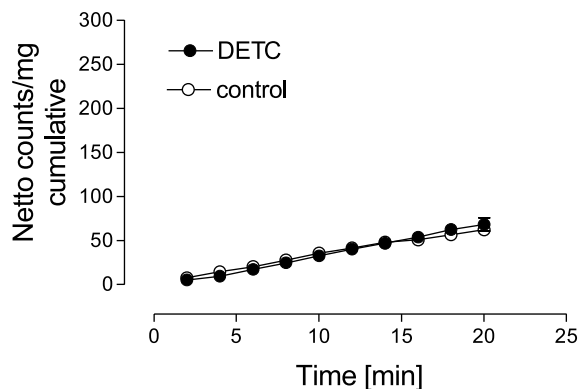
### 3.2. Effect of DETC on XO-induced superoxide generation

To evaluate an interference of DETC with superoxide detection by lucigenin, we first monitored generation of superoxide by xanthine/XO (X/XO) with and without DETC. Increasing concentrations of xanthine correlated with the rate of superoxide production (Fig. 2A) reaching a maximum value of  $9861 \pm 937$  counts at 2.4  $\mu$ M xanthine. This was almost completely abolished by DETC (Fig. 2B).

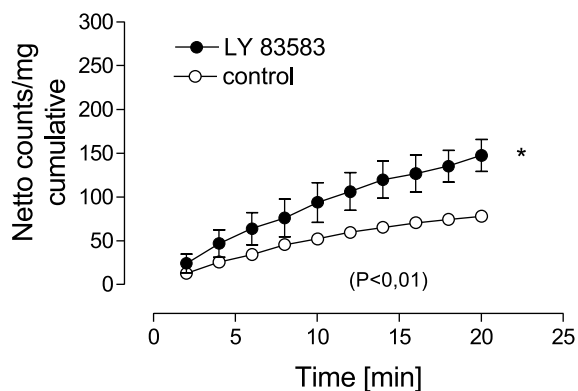
### 3.3. Effect of DETC on oxidation of adrenaline by X/XO

To confirm the inhibition of XO by DETC, we monitored

A.



B.



C.

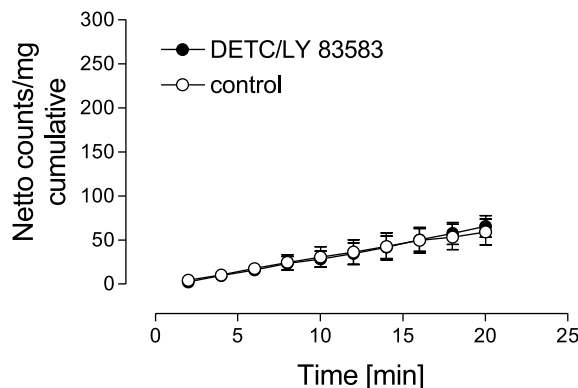


Fig. 1. Bioavailability of superoxide in rat aortic rings preincubated with 10 mM DETC (A), 10  $\mu$ M LY83583 (B) and 10 mM DETC plus 10  $\mu$ M LY83583 (C). Superoxide was measured by lucigenin chemiluminescence. Each point represents the mean ( $\pm$  S.E.M., occasionally smaller than symbols) of three different experiments using three different rats ( $*P < 0.01$ , ANOVA).

the oxidation of adrenaline by different concentrations of  $\text{KO}_2$  and found no effect of 1 mM DETC (Fig. 3A), while the inhibitory effect of SOD on adrenochrome formation was almost blunted by DETC suggesting that DETC inhibits SOD

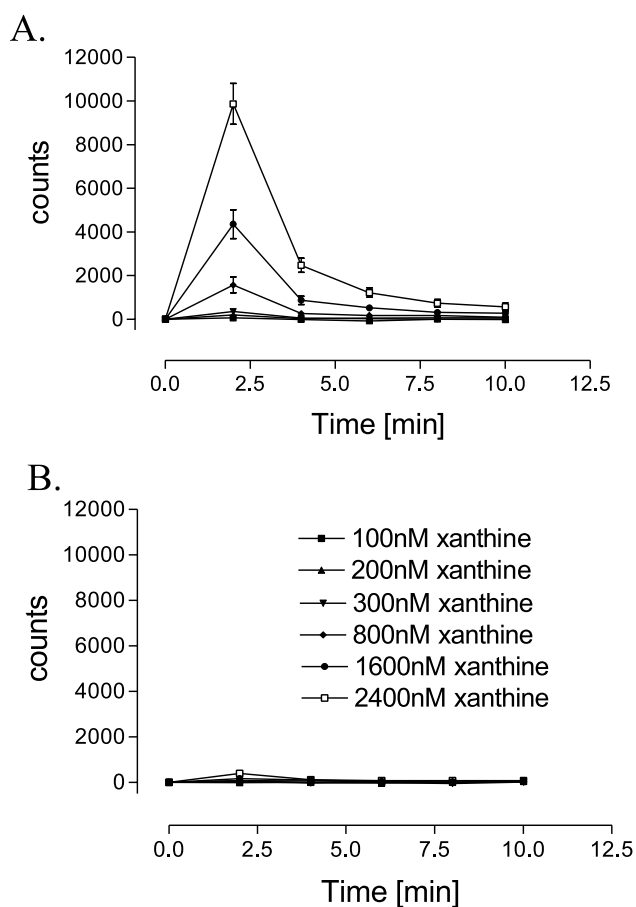


Fig. 2. Effect of 10 mM DETC on superoxide production by the X/XO system. A: Production of superoxide during incubation of XO with increasing concentrations of xanthine (0.1–2.4  $\mu$ M). B: Production of superoxide during incubation of XO with increasing concentrations of xanthine (0.1–2.4  $\mu$ M) in the presence of 10 mM DETC.

activity. Furthermore, DETC concentration dependently inhibited adrenochrome formation induced by X/XO (Fig. 3C and D). These data indicate that 1 mM DETC, a concentration often used to inhibit SODs, can completely inhibit the catalytic activity of XO.

#### 3.4. Effect of DETC and LY83583 on vascular sGC activity

Changes of NO-stimulated sGC activity were used to measure consequences of increased superoxide in aortic rings treated with DETC and LY83583. Contrary to DETC, LY83583 strongly decreased the SNAP-dependent activation of sGC (Fig. 4). These results indicate that superoxide strongly inhibits sGC activity. In addition, these data confirm that DETC does not increase vascular superoxide generation.

#### 3.5. Effect of DETC and LY83583 on vascular sGC expression

The LY83583-induced decrease of sGC activity might be explained by a reduction of sGC expression. However, Western blot analysis showed that LY83583 induced a significant increase of sGC expression (Fig. 5) suggesting that inhibition of sGC activity by intracellular superoxide induces a compensatory increase of enzyme expression. Again DETC had no effect which further confirms that DETC does not increase vascular superoxide production in rat aorta.

## 4. Discussion

We investigated the effects of DETC, a well-known inhibitor of SOD, on vascular superoxide production. Our new finding is that DETC can completely inhibit the catalytic activity of vascular XO. Furthermore, DETC did not affect basal vascular superoxide production but abolished the LY83583-induced increase of superoxide. Cellular effects induced by increased vascular superoxide production such as a reduced expression and activity of sGC were not initiated by DETC. These data suggest that DETC inhibits oxidoreductase enzymes such as XO and those reducing LY83583 in rat aorta.

DETC is a metal ion-chelating agent which is known as an effective inhibitor of SODs [3]. Other known actions of DETC include the inhibition of NF $\kappa$ B and its ability to trap NO [4,5]. Here we report that DETC is also an inhibitor of vascular XO. In experiments with rat aortic rings, DETC did not increase vascular superoxide. Furthermore, DETC completely abolished the increase of vascular superoxide induced by treatment with LY83583. There are many different mechanisms which might explain this unexpected activity of DETC. Among these, inhibition of superoxide generation from LY83583 and a scavenger effect for readily generated superoxide seem to be particularly interesting.

Monitoring lucigenin signals during incubation of XO with increasing concentrations of xanthine showed a strong inhibitory activity of DETC. Further experiments demonstrated a concentration-dependent inhibition of the X/XO-induced oxidation of adrenaline to adrenochrome by DETC, while the oxidation induced by KO<sub>2</sub> was not affected. These data strongly indicate that DETC inhibits the catalytic activity of XO. However, it is rather unlikely that this enzyme, which introduces oxygen residues, contributes to the enzymatic reduction of LY83583 in intact aortic rings. In general, it is assumed that the semiquinone radical resulting from a one-electron reduction of LY83583 transfers its electron to molecular oxygen and induces superoxide formation. Indeed, prior reports demonstrated that LY83583 can be processed by different NADPH-dependent enzymes. For example, Kumagai et al. reported the generation of superoxide from LY83583, as detected by electron spin resonance, in incubation mixtures containing purified neuronal NO synthase or P450 reductase [15]. Both enzymes are present in vascular endothelial and/or smooth muscle cells. These data are consistent with another study showing that a variety of novel quinone compounds potentially inhibit endothelial NO synthase by interacting with the reductase domain of this enzyme [16]. Other investigators were able to demonstrate superoxide generation from LY83583 mediated by quinone oxidoreductase [17].

Unexpectedly, we did not observe any effect of DETC on superoxide production in rat aorta, although inhibition of SOD and trapping of NO should result in an increase of vascular superoxide. It was indeed demonstrated that DETC can slightly increase the lucigenin chemiluminescence signal in atherosclerotic rabbit aorta [6,7]. This small difference to our results might be explained by species differences. There are multiple sources of vascular superoxide in normal arteries such as NADPH oxidase, cyclooxygenase, XO, myeloperoxidase and non-enzymatic generation by the transfer of electrons from coenzyme Q to molecular oxygen during adenosine triphosphate (ATP) synthesis in mitochondria [1,18,19]. It is

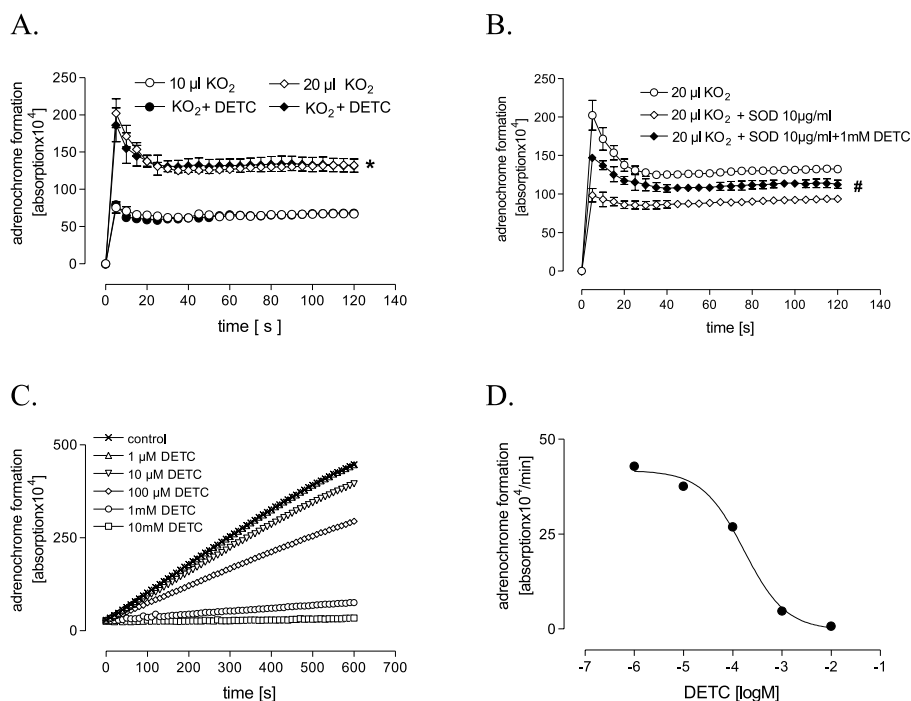


Fig. 3. Effect of 10 and 20  $\mu$ l of a saturated  $KO_2$  solution on formation of adrenochrome. A: DETC (1 mM) has no effect on adrenochrome formation. B: SOD inhibits adrenochrome formation by  $KO_2$  and this effect is significantly reduced by the SOD inhibitor DETC (1 mM). C: Inhibitory effect of DETC on superoxide production by the X/XO system. D: Concentration-dependent inhibition of superoxide generation by DETC (\* $P < 0.001$  for 10  $\mu$ l  $KO_2$  vs. 20  $\mu$ l  $KO_2$ , # $P < 0.05$  for  $KO_2$ +SOD vs.  $KO_2$ +SOD+DETC).

quite possible that the contribution of each of these sources to the total vascular superoxide production varies among different species and tissues. In addition, DETC-induced inhibition of superoxide production by XO in rat aorta might counterbalance its inhibitory effect on SOD so that the net effect is no change in vascular superoxide production.

This net effect on superoxide production is consistent with the finding that DETC had no effect on the expression and activity of vascular sGC. Previous investigations have shown a negative feedback loop between sGC activity and expression in isolated smooth muscle cells [20]. These results have been confirmed and extended to the effects of superoxide and per-

oxynitrite on this feedback loop in isolated vessels and in vivo by several investigations in our lab [10,11]. These oxygen derived radicals inactivate sGC and initiate its expression. Furthermore, not only increased superoxide but also pathologic alterations associated with an increase of vascular bioavailability of reactive oxygen species such as atherosclerosis inhibit the catalytic activity of vascular sGC and markedly increase its expression [10]. Thus, the activity and expression of vascular sGC appear to be a sensitive biomarker for vascular oxidative stress.

In summary, we found that the SOD inhibitor DETC had no effect on basal vascular superoxide bioavailability in rat aorta, possibly because DETC inhibits both vascular superoxide generation by blocking the catalytic activity of oxidoreductase enzymes such as XO and those reducing LY83583 in rat aorta and vascular superoxide degradation by inhibition of SOD.

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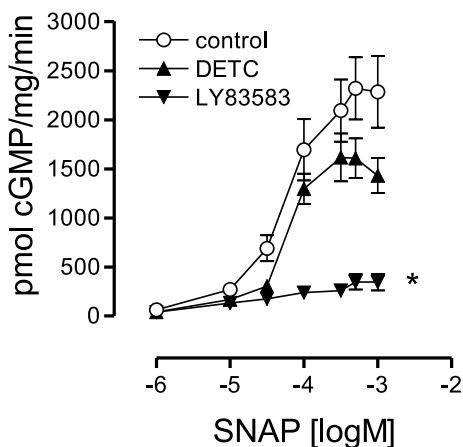


Fig. 4. Specific activity of sGC in supernatants of rat aortic rings incubated with vehicle, DETC (10 mM) and the superoxide radical generator LY83583 (10  $\mu$ M) for 4 h. DETC had no significant effect, while increased superoxide generation induced by LY83583 (see Fig. 1) strongly reduced the specific activity of sGC.

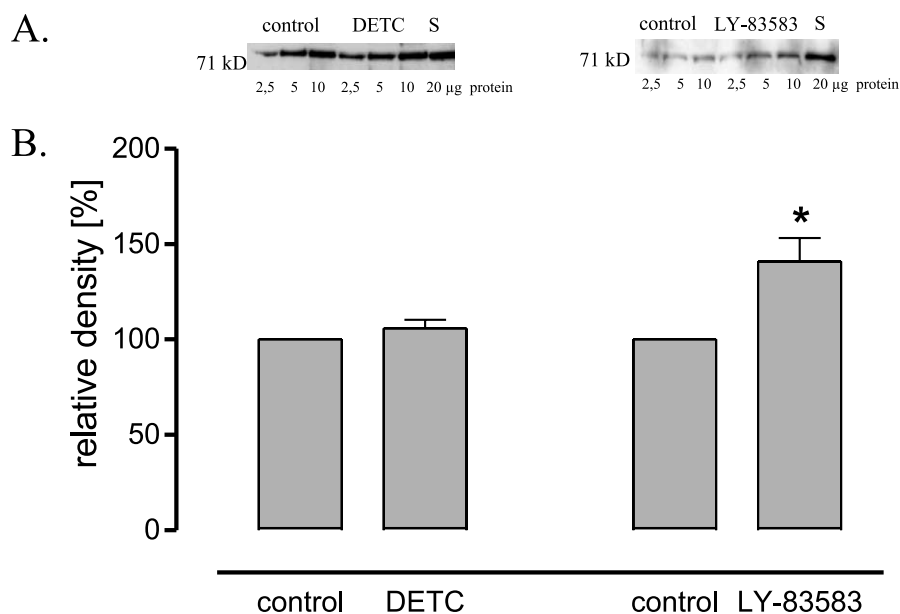


Fig. 5. Expression of sGC in rat aortic rings incubated with vehicle, DETC (10 mM) and the superoxide radical generator LY83583 (10  $\mu$ M) for 4 h. DETC had no significant effect, while increased superoxide generation induced by LY83583 (see Fig. 1) strongly reduced the specific activity of sGC. A representative Western blot (A) and the summary of six different incubation experiments (B) are shown for each experimental condition.

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