JPET Fast Forward. Published on February 7, 2006 as DOI: 10.1124/jpet.105.097634

Title Page

Lipopolysaccharide induces epithelium- and prostaglandin E_2-dependent relaxation of mouse isolated trachea through activation of COX-1 and COX-2

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JPET Fast Forward. Published on February 7, 2006 as DOI: 10.1124/jpet.105.097634
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Running Title Page

Running Title: LPS-induced relaxation of mouse airways

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Number of Text Pages: 29

Number of Tables: 0

Number of Figures: 7

Number of References: 40

Number of Words in Abstract: 250

Number of Words in Introduction: 405

Number of Words in Discussion: 1507

Non-standard Abbreviations: $cPLA_2$, cytosolic phospholipase A_2; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; PAR, protease-activated receptor; SLIGRL, single letter amino acid code for the PAR_2-peptide agonist Ser-Leu-Isoleu-Gly-Arg-Leu-NH_2; TLR, Toll-like receptor.

Recommended Section Assignment: Inflammation and Immunopharmacology

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Abstract

Lipopolysaccharide (LPS), a Toll-like receptor 4 (TLR4) agonist, causes airway hyperreactivity through nuclear factor-kappa B (NF-κB). Since NF-κB induces cyclooxygenase-2 (COX-2) to increase synthesis of prostaglandins (PGs), including the potent airway anti-inflammatory and smooth muscle relaxant, PGE_2, we investigated whether LPS causes short-term PGE_2-dependent relaxation of mouse isolated trachea. In rings of trachea contracted submaximally with carbachol, LPS caused slowly-developing, epithelium-dependent relaxations that reached a maximum within 60 min. Fluorescence immunohistochemistry revealed TLR4-like immunoreactivity localised predominantly to the epithelium. The LPS antagonist polymixin B, the non-selective COX inhibitor indomethacin, the selective COX-1 and COX-2 inhibitors 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-(trifluoromethyl)-1H-pyrazole (SC560) and 4-[5-(4-chlorophenyl)-1-(trifluoromethyl)-1H-pyrazol-1-yl]-benzenesulfonamide (SC236) respectively, the transcription inhibitor actinomycin D, the translation inhibitor cycloheximide, the p38 mitogen-activated protein kinase (p38 MAPK) inhibitor [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imadazole] (SB203580) and a combination of the mixed DP/EP1/EP2 receptor antagonist 6-Isopropoxy-9-xanthone-2-carboxylic acid (AH6809) and the EP4 receptor antagonist L-161982, all abolished relaxation to LPS, giving instead slowly-developing, small contractions over 60 min. The cytoplasmic phospholipase A_2 ($cPLA_2$) inhibitor 1,1,1-Trifluoro-6Z,9Z,12Z,15Z-heneicosateraen-2-one ($AACOCF_3$) significantly ($p < 0.05$) inhibited the relaxation to LPS, while the NF-κB proteasomal inhibitor Z-Leu-Leu-Leu-aldehyde (MG-132) had no affect on the relaxation in the first 20 min, after which it reversed the response to a contraction. In

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conclusion, our data indicate that LPS activates airway epithelial TLR4 to cause release of PGE_2 and subsequent EP2 and EP4 receptor-dependent smooth muscle relaxation. Activation of both COX-1 and COX-2 appears to be essential for this novel response to LPS, which also involves $cPLA_2$, p38 MAPK, NF-κB and an unidentified NF-κB-independent, labile regulatory protein.

Introduction

Lipopolysaccharide (LPS), a product of the Gram-negative bacterial cell wall, potently stimulates the innate immune system, eliciting both pro- and anti-inflammatory responses (Ulevitch and Tobias, 1995). Mediation of cellular activation in response to LPS is known to occur through Toll-like receptor 4 (TLR4), a member of the Toll receptor family (Tapping et al., 2000). TLR4 shares a common pro-inflammatory signal transduction pathway with other pro-inflammatory mediators such as IL-1, which leads to the nuclear translocation of NF-κB and transcription of various genes (Vogel et al., 1999). Additionally, LPS induces signalling via c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal regulated kinase 1/2 (ERK1/2) (Han et al., 1994; Liu et al., 1994; Hambleton et al., 1996). Activation of NF-κB causes enhanced expression of genes encoding inflammatory cytokines, acute phase proteins, immunoreceptors and chemokines important in the recruitment of neutrophils, eosinophils, macrophages and lymphocytes (Hirano, 1997; Becker et al., 2000). For example, the gene for cyclooxygenase-2 (COX-2) has NF-κB binding sequences in its promoter region, which are critical for transcriptional activation (Schmedtje et al., 1997). Thus, enhanced COX-2 activity induced by LPS in rat alveolar macrophages, the major cell type thought to mediate most of the responses to inhaled LPS (Koay et al., 2002), is due to the selective increase in levels of COX-2 protein, suggesting that upregulation of COX-2 is responsible for increased synthesis of prostaglandins (PG) and thromboxanes (Lee et al., 1992; Phillips et al., 1993). Long-term pre-treatment of either whole animals or isolated tissues with LPS is known to induce

alterations in airway reactivity to a variety of bronchoconstrictors, including histamine and bradykinin (Pauwels et al., 1990; Bachar et al., 2004; Johnston et al., 2004). Also, LPS has been shown to cause a decrease in the forced expiratory volume in 1 s (FEV_1) in asthmatic patients (Michel et al., 1996). By contrast, short-term (30 min) pre-treatment of guinea pig isolated trachea with LPS has been shown to reduce contractions to methacholine and histamine largely in an epithelium-independent manner (Fedan et al., 1995). Here we show in the mouse isolated trachea that LPS induces acute, slowly-developing, epithelium-dependent relaxation that requires the simultaneous activation of COX-1 and COX-2 and is mediated by both EP2 and EP4 prostaglandin E_2 (PGE_2) receptors. This novel response to LPS appears to be regulated by p38 MAPK, $cPLA_2$, NF-κB and another, as yet unidentified, labile protein and may have an important role in airway barrier defence.

Methods

Animals. This study was conducted under guidelines set by the National Health and Medical Research Council of Australia and was approved by the University of Melbourne's Animal Welfare Committee. Balb/C mice (male, 8 weeks old) were killed by overdose of pentobarbitone sodium (240 $mg.kg^{-1}$ i.p.) before removing the trachea.

Tension recording in isolated tissues. Trachea were cut into two rings and mounted on two stainless steel hooks in organ baths containing Krebs solution (composition (mM): NaCl 118; KCl 4.7; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25; $CaCl_2$ 2.5; glucose 11), continuously aerated with 95% O_2 and 5% CO_2 and maintained at 37°C. One hook was connected to a micrometer adjustable support, the other to a force transducer (Model FT.03, Grass Instruments, Massachusetts, U.S.A.) linked via an amplifier to a chart recorder (Model 330, W & W Scientific Instruments, Basel, Switzerland) for continuous recording of changes in isometric tension. Tracheal rings were allowed a 20 min equilibration period before being placed under 0.5 g of passive force. When baseline levels of passive force stabilised, maximum contractions (F_{max}) to acetylcholine (ACh, 10 μM) were determined. Tissues were then washed with Krebs solution and allowed to return to baseline levels of passive force. Nifedipine (0.3 μM) was then added to reduce spontaneous smooth muscle activity (Cocks et al., 1999) and tissues contracted with titrated concentrations of carbachol (50 - 130 nM) until active force reached approximately 30% F_{max}. LPS (*E. coli* strain O127:B8, 10 $\mu g.ml^{-1}$) was added when the active contraction to carbachol reached a stable plateau. Drugs used to determine the

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mechanisms of LPS relaxation were added at least 40 min prior to LPS ($AACOCF_3$, 10 μM; AH6809, 3 μM; indomethacin, 3 μM; L-161982, 1 μM; L732138, 0.1 μM; MG-132, 10 μM; SB203580, 10 μM; SC236, 1 μM; SC560, 0.3 μM), except polymixin B (10 $\mu g.ml^{-1}$), which was incubated with LPS for 10 min before being added to the organ bath, to allow the antagonist to bind with LPS. Actinomycin D (2 μM) and cycloheximide (10 μM) were added 40 min prior to LPS. None of the drugs used altered the concentration of carbachol required to reach 30% F_{max} (data not shown), indicating that none of these compounds altered smooth muscle reactivity. As well as using the neurokinin-1 receptor (NK_1R) antagonist L732128 (0.1 μM), any role for the release of the potent epithelium-dependent relaxant, substance P (Szarek et al., 1998) or any other neurotransmitters from sensory nerves, in LPS-induced relaxation was further examined by treating the trachea with capsaicin (10 μM) for 1 h prior to addition of LPS. Similarly, any role for LPS-induced release of activators of protease-activated receptor-1 (PAR_1) and PAR_2 (Cocks et al., 1999) such as mast cell tryptase (Cocks and Moffatt, 2000) was examined by desensitising these receptors with trypsin (Cocks et al., 1999). This involved repeated additions of trypsin ($3\ U.ml^{-1}$) to cause maximum relaxation followed by recovery of active force to the pre-stimulation level (30% F_{max}) without washing out the trypsin, until no relaxation was observed, at which point LPS was then added. The epithelium was removed from the trachea according to the method of Cocks et al (1999). Briefly, warm Krebs solution containing 1% Triton X-100 was gently flushed into the trachea *in situ* before it was dissected. To test the functional effectiveness of epithelium removal, responsiveness to the PAR_2 agonist SLIGRL (Cocks et al., 1999) was tested prior to the addition of LPS.

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Immunohistochemistry. Mouse trachea were frozen in OCT (Sakura Finetek, California, U.S.A.) and 5 μm serial cryostat sections were cut and air-dried at room temperature for several hours, then fixed in acetone for 10 min. Sections were blocked with normal serum and then labelled with goat anti-mouse TLR4 antibody (1/50). Primary antibody was bound with biotinylated donkey anti-goat IgG (1/500) and fluorescently labelled with streptavidin-conjugated Texas Red (1/500). Sections were microscopically examined using the appropriate filter.

Materials. Acetylcholine, capsaicin, carbachol, cycloheximide, indomethacin, lipopolysaccharides (*E.coli* strain O127:B8), substance P, Triton X-100, trypsin (Sigma, Missouri, U.S.A.); nifedipine (Sapphire Bioscience, N.S.W., Australia); $AACOCF_3$, AH6809, L732138 and MG-132 (Tocris, Missouri, U.S.A.); actinomycin D and SB203580 (A.G. Scientific, California, U.S.A.); SC236 and SC560 (Cayman Chemicals, Michigan, U.S.A.); polymixin B (Invivogen, San Diego, U.S.A.); Streptavidin-Texas Red (Vector Laboratories, California, U.S.A.), all antibodies (Santa Cruz Biotechnology, California, U.S.A.) and PAR_2-activating peptide SLIGRL (single letter amino acid code and amidated at the carboxyl terminal, Auspep, Victoria, Australia). L-161982 was a kind gift from Dr. Robert Young (Merck-Frosst Canada Ltd, Quebec, Canada). Acetylcholine, carbachol, indomethacin, L-161982, lipopolysaccharides, polymixin B, substance P, SLIGRL and trypsin were made up as concentrated stock solutions in distilled water. $AACOCF_3$, capsaicin, L732138, nifedipine, SB203580, SC236 and SC560 were made up in 100% ethanol. Actinomycin D, AH6809, cycloheximide and MG-132 were made up in

dimethyl sulphoxide (DMSO). Of the above vehicles, only DMSO had an affect on LPS-induced relaxation of mouse isolated trachea, causing a small, but significant ($p < 0.05$) inhibition of the response at 20 min (Figure 5).

Data Analysis. Responses (mean ± S.E.M) are expressed as percentage relaxations or contractions from the 30% F_{max} level of active force. Comparisons between groups were made using either unpaired t tests or one-way ANOVA, with Bonferroni's corrections for multiple comparisons. $p < 0.05$ was accepted as being significant.

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Results

Epithelium-dependent relaxation to LPS. LPS purified by either phenol or trichloroacetic acid (TCA) extraction caused similar delayed onset, slowly-developing relaxations that reached maxima of 76.4 ± 2.4% and 77.0 ± 6.9% respectively at 60 min (Figure 1). The spontaneous relaxation of control tissues was 23.6 ± 7.3% at 60 min (Figure 1). Therefore, all subsequent experiments were carried out using LPS purified by either TCA or phenol. The response to LPS was abolished following *in situ* perfusion of the trachea with 1% Triton X-100. We have previously demonstrated histologically that this procedure removes most of the epithelium in mouse trachea and leaves the underlying smooth muscle structurally normal (Cocks et al., 1999). Success of this method to remove most of the epithelium whilst not damaging the smooth muscle was determined functionally with the epithelium-dependent PAR_2 peptide, SLIGRL (Cocks et al., 1999). Thus, in control and Triton X-100-treated tissues SLIGRL (10 μM) caused maximum relaxations of 87.4 ± 3.9% and 18.6 ± 3.9% respectively (Figure 2). Using a mouse specific TLR4 antibody, extensive TLR4-specific immunofluorescence was localised to the epithelium, whilst other structures such as the cartilage and smooth muscle layers were devoid of TLR4-like staining (Figure 3). Control sections not treated with the primary TLR4 antibody exhibited only weak autofluorescence of the epithelium (Figure 3).

Mechanism of LPS-induced relaxation. Incubation of LPS with the LPS antagonist polymixin B (10 $\mu g.ml^{-1}$) 10 min prior to addition to the tissue, abolished the relaxation

to LPS and instead caused a slowly-developing, variable contraction of 16.4 ± 14.5% at 60 min (Figure 4). Polymixin B alone did not affect the tone of the tissue (data not shown). The sensory nerve toxin, capsaicin, the selective NK_1R antagonist, L732138 and desensitisation of PARs with trypsin had no effect on LPS-induced relaxations that reached maxima of 79.0 ± 2.4%, 86.3 ± 5.2% and 87.2 ± 5.1% respectively at 60 min (Figure 4). These results indicate that the relaxation to LPS was unlikely to involve the release of sensory nerve transmitters like substance P or activators of PAR_1 and PAR_2 such as mast cell tryptase (Cocks and Moffatt, 2000). The relaxation to LPS was reversed to similar, slowly-developing contractions by the non-selective COX inhibitor, indomethacin (3 μM) as well as the COX-1 and COX-2 selective inhibitors, SC560 (0.3 μM) and SC236 (1 μM), respectively (Figure 4). The mixed DP/EP1/EP2 receptor antagonist, AH6809 (3 μM) and the specific EP4 receptor antagonist, L-161982 (1 μM), both trended to inhibit the response to LPS, with relaxation reaching maxima of 52.8 ± 18.4% and 46.0 ± 4.8% respectively at 60 min. However, these values were not significantly different from the 60 min control response (77.3 ± 6.2%; Figure 4). In contrast, AH6809 and L-161982 added together reversed the LPS-induced relaxation to a slowly-developing contraction of 10.8 ± 4.4% at 60 min (Figure 4). Together, these results indicate that LPS causes release from the epithelium of PGE_2, a known potent airway smooth muscle relaxant (Lan et al., 2001; Vancheri et al., 2004).

The p38 MAPK inhibitor, SB203580 (10μM) also reversed the LPS-induced relaxation to a slowly-developing contraction of 13.3 ± 8.6% at 60 min (Figure 5), whereas the $cPLA_2$ inhibitor $AACOCF_3$ (10 μM) partially, but significantly ($p < 0.05$), inhibited the

relaxation to LPS (60 min maximum response 47.1 ± 6.3% vs. control 75.2 ± 7.1%; Figure 5). MG-132 (10 μM), a NF-κB proteasomal inhibitor, had no effect on the relaxation to LPS over the first 20 min of the response, but after this time the response was reversed to a contraction (Figure 5). Finally the transcription inhibitor, actinomycin D (2 μM) and the translation inhibitor, cycloheximide (10 μM) both abolished the relaxation to LPS when added 40 min prior to LPS, giving instead slowly-developing contractions of 14.0 ± 3.7% and 10.5 ± 5.7% respectively at 60 min (Figure 6). Actinomycin D (2 μM), however, had no effect on the maximum relaxation to substance P (control 98.1 ± 1.2%; actinomycin D 98.4 ± 1.0%; n = 5). Neither actinomycin D nor cycloheximide had any effect on the tone of tissue (data not shown).

Effect of COX-1 and COX-2 inhibitors on relaxations to substance P and SLIGRL. Cumulative relaxation curves for the known epithelium-dependent relaxants, the NK_1R agonist substance P (Szarek et al., 1998) and the PAR_2-selective peptide agonist, SLIGRL (Cocks et al., 1999) were markedly inhibited by either SC560 (0.3 μM) or SC236 (1 μM). For SLIGRL, combined SC560 and SC236 treatment trended to increase the degree of inhibition caused by either inhibitor alone but this failed to reach significance (Figure 7). In order to more carefully assess the apparent interaction between COX-1 and COX-2 underlying these responses, we re-examined the effect of the selective COX-1 and COX-2 inhibitors on the relaxations to combined substance P and SLIGRL additions. Under these conditions of increased stimulus strength, each COX inhibitor caused an approximately 100-fold shift to the right of the control curve, which was abolished by the combined addition of both inhibitors (Figure 7).

Discussion

This study suggests that the TLR4 agonist, bacterial LPS, causes acute, sustained release of PGE_2 from mouse airway epithelial cells. Thus, using a sensitive smooth muscle contractility bioassay we found LPS caused epithelium-dependent relaxation of the mouse trachea and that the response was blocked by inhibitors of LPS, p38 MAPK, NF-κB, $cPLA_2$, COX-1 and COX-2, EP2 and EP4 receptors as well as actinomycin D and cycloheximide. Given the importance of PGE_2 in airway barrier defence (Vancheri et al., 2004), we propose that TLR4-induced release of PGE_2 plays a fine-tuning, regulatory role in the both the innate and acquired immune responses to bacterial infection in the lungs that includes acute bronchodilatation.

The development of airway hyperreactivity after LPS administration to both humans (Michel et al., 1992) and animals (Pauwels et al., 1990; Held and Uhlig, 2000) is well documented. To our knowledge, only one previous study has shown acute inhibitory effects of LPS on airway reactivity, albeit indirectly. Thus, Fedan et al (1995) showed that acute LPS treatment of perfused guinea pig trachea in vitro depressed contractility to both extraluminally- and intraluminally-applied histamine and methacholine, which they concluded was due mainly to a direct depressant effect on the trachealis smooth muscle. This is in contrast to our study, where the direct relaxation of mouse trachea to LPS was entirely epithelium-dependent. Apart from different species of animals and serotypes of LPS, reasons for the apparent discrepancy between our study and that of Fedan et al (1995) remain unknown.

Epithelium-dependent airway smooth muscle relaxations caused by PAR_1 and PAR_2 peptide agonists (Cocks et al., 1999), previously thought to be mediated by constitutive COX-2 (Lan et al., 2001), most likely involve both COX-1 and COX-2 isoforms (Kawabata et al., 2004), similar to our finding here for LPS. Kawabata et al (2004) suggested that, like COX-1, COX-2 is constitutively expressed in the mouse airways and that both isoforms act sequentially to produce PGE_2 in response to PAR activation in a manner analogous to that suggested for PAR_1-induced Cl^- secretion in cell monolayers of the intestinal cell line SCBN (Buresi et al., 2002). One way such a pathway may function is that prostanoids formed by one of the COX isoforms enhance activity of the other, as suggested by Yamada et al (2004). We, however, favour a more simple explanation that both COX isoforms need to be activated in parallel in order to generate sufficient PGE_2 release from the epithelium to cause relaxation of the underlying smooth muscle. This view is supported by our finding that the concentration-relaxation curve to a combination of two high efficacy, epithelium-dependent relaxants, the PAR_2 agonist peptide SLIGRL (Cocks et al., 1999) and the NK_1R agonist substance P (Szarek et al., 1998) was right-shifted approximately 100-fold by either SC560 or SC236 (the same inhibitors used to abolish LPS-mediated relaxation) alone and abolished when both antagonists were added together. Similar relaxation curves to SLIGRL and SP alone were, like the response to LPS, all but abolished by each COX isoform-selective inhibitor. Assuming selective and effective inhibition of COX-1 and COX-2 by SC560 and SC236 respectively, our findings with substance P and SLIGRL indicate that COX-1 and COX-2 produce similar amounts of PGE_2 per unit stimulation, but in amounts insufficient to activate EP2 and

EP4 receptors on smooth muscle and cause relaxation. Relaxations due to only one COX isoform become evident only when the stimulus strength is increased such as was observed here with combined NK_1R and PAR_2 activation.

The relatively rapid (minutes) relaxation to LPS, together with the requirement for each isoform to be activated in order to observe the response, further suggests that, like COX-1, COX-2 is constitutively expressed in the mouse trachea. COX-2 has been found in the epithelium of airways without clinical or histological evidence of airway inflammation (Watkins et al., 1999) and Demoly et al (1998) reported 'constitutive' expression of COX-2 in human nasal respiratory epithelium. Recent studies have also demonstrated constitutive expression of COX-2 in brain and kidney (Harris et al., 1994; Breder et al., 1995). Given that the LPS-induced relaxation was abolished by inhibitors of both gene transcription (actinomycin D) and mRNA translation (cycloheximide) and that both COX isoforms were required to be activated for the response to occur, it remains possible that COX-2 is both rapidly upregulated and degraded i.e. it undergoes high turnover. This seems unlikely, however, since similar COX-2 (and COX-1) -dependent relaxations to substance P were unaffected by actinomycin D. The proteasomal (and thus NF-κB) inhibitor, MG-132 (Chen et al., 1997), however, did block the latter phase of the response to LPS, suggesting some role for NF-κB-dependent gene transcription. Given NK_1R-mediated PGE_2 release in the same tissue does not depend on transcription, we suggest that NF-κB is involved in TLR4-mediated PGE_2 release by LPS, but not via up-regulation of COX-2. Also, both the NF-κB-independent and -dependent early and late phases respectively of the LPS response most likely involve events upstream from COX-

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1, COX-2 and PGE_2 synthesis since downstream events, including actions of PGE_2 on the smooth muscle, would be expected to be similar for TLR4 and NK_1R.

The rate-limiting step in the biosynthesis of PGE_2 is the liberation of arachidonic acid through the activity of cytosolic phospholipase A_2 ($cPLA_2$) that is regulated by Ca^{2+}-dependent translocation from the cytosol to membranes and phosphorylation, which increases the intrinsic activity of $cPLA_2$ by 2 - 4 fold (Lin et al., 1993). Our results with the cytosolic phospholipase A_2 ($cPLA_2$) inhibitor $AACOCF_3$, indicate that $cPLA_2$ is involved in LPS-induced PGE_2 release from mouse airway epithelium. Lin et al (1993) demonstrated the importance of phosphorylation of $cPLA_2$ by MAPK in its activation, presenting a link between the MAPK pathway and COX activity. Furthermore, Buresi et al (2002) suggested that for PAR_1 activation in intestinal epithelium the ERK 1/2 MAPK pathway and COX pathway act in series, as they were able to block $cPLA_2$ phosphorylation using a MEK inhibitor, showing $cPLA_2$ to be dependent on the ERK 1/2 MAPK pathway. Our findings with SB203580 suggest that p38 MAPK is the main kinase involved in LPS-induced release of PGE_2 in the airway epithelium, in agreement with Kawabata et al (2004), who similarly reported p38 MAPK was involved in COX-dependent relaxation of mouse trachea to PAR_1 and PAR_2 activation.

Regardless of the exact signalling pathways involved in LPS-induced PGE_2 release from airway epithelial cells, the reasons why TLR4 is coupled to both COX-1 and COX-2 and why the ensuing process of PGE_2 release appears to be so tightly regulated are intriguing questions. TLRs are expressed on barrier defensive cells like airway epithelium primarily

to detect invading pathogens such as bacteria, viruses and fungi (Pasare and Medzhitov, 2005). For TLR4, detection of bacterial LPS results in an early, rapid and predominantly TNF-α-mediated neutrophilia to help kill the invading bacteria. Concomitant release of PGE_2 at first appears to be counter-productive to this essential innate defence mechanism since airway neutrophil trafficking to LPS inhalation in conscious mice is inhibited by PGE_2 applied exogenously (Goncalves de Moraes et al., 1996) or released endogenously by PAR_2 agonists (Moffatt et al., 2002). However, a case may be made for a beneficial role for LPS-induced PGE_2 release during bacterial infections if the time course of release is taken into account. We have shown here using a sensitive PGE_2 bioassay - tracheal smooth muscle relaxation (Cocks et al., 1999; Lan et al., 2001; Kawabata et al., 2004) - that release of PGE_2 from the epithelium in response to LPS appears to accumulate slowly to an apparent maximum rate over 60 min. Therefore, timing of PGE_2 synthesis and release versus release of pro-inflammatory cytokines may be vital for coordinating neutrophilia in response to LPS. Smooth muscle relaxation and thus dilatation of the inflamed airways would similarly be beneficial by helping maintain airflow in a setting of infection. Another possible beneficial role for delayed TLR4-mediated PGE_2 release from airway epithelium could be to help prime the acquired immune system towards an appropriate antibody response since, like certain pro-inflammatory cytokines, PGE_2 is a key mediator of dendritic cell activation (Kalinski et al., 1998). Again, timing between LPS-mediated PGE_2 release and release of other co-modulators like IL-6 and IL-8 (Medzhitov et al., 1997) may be vital for an appropriate delayed antibody response. It should, however, be noted that long-term exposure to LPS has been shown to cause airway epithelial cell damage (Folkerts et al., 1988) and reduction in PGE_2 release

(Folkerts et al., 1989). Therefore, we suggest that any immunomodulatory effects of LPS occur over a relatively short term following initial exposure.

In conclusion, epithelium-dependent relaxation to LPS through TLR4 in mouse isolated trachea, appears to involve a novel p38 MAPK- and $cPLA_2$-dependent pathway that leads to co-activation of constitutive COX-1 and COX-2 and release of PGE_2. Also, although NF-κB may have a regulatory role in the later stages of this acute response to LPS, it appears to be predominantly controlled by another labile, as yet unidentified protein. We speculate that during bacterial infections, such tight control allows TLR4 to fine tune PGE_2-dependent counter-inflammatory responses, including airway smooth muscle relaxation.

Acknowledgments

We thank Dr. Robert Young of Merck Frosst Canada Ltd for providing the EP4 antagonist L-161982.

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Footnotes

This work was supported by the National Health and Medical Research Council (NHMRC) of Australia.

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Figure Legends

Figure 1. LPS-induced relaxation of mouse isolated trachea. LPS purified by two different techniques (phenol and trichloroacetic acid; TCA) were used (both at 10 $\mu g.ml^{-1}$) and the responses over time compared to normal (control) loss of tone. Values (means ± S.E.M from n = 7 – 25) are expressed as percentages of pre-LPS levels of active force induced by carbachol.

Figure 2. Representative copies of original chart recordings showing relaxations to the PAR_2 peptide, SLIGRL (10 µM) and LPS (10 $\mu g.ml^{-1}$) in (A) a control preparation of mouse isolated trachea and (B) one treated with Triton X-100 to remove the epithelium. Tissues were contracted with carbachol to 30% maximum contraction to acetylcholine (30% F_{max}). (C) Group data from n = 5 experiments described in (A) and (B).

Figure 3. Hematoxylin and eosin staining of mouse trachea (A) clearly showing the structure of the epithelium and cartilage and fluorescence immunohistochemical staining for TLR4 in the mouse trachea with (B) primary antibody showing TLR4 immunoreactivity in the epithelium and endogenous biotin in the cartilage and (C) without primary antibody, showing endogenous biotin in the cartilage and low-level autofluorescence of the epithelium.

Figure 4. The effects of (A) the sensory nerve toxin capsaicin (10 µM for 1 h, n = 5), desensitisation with trypsin (n = 8), the NK_1R antagonist L732138 (0.1 µM, n = 3), the

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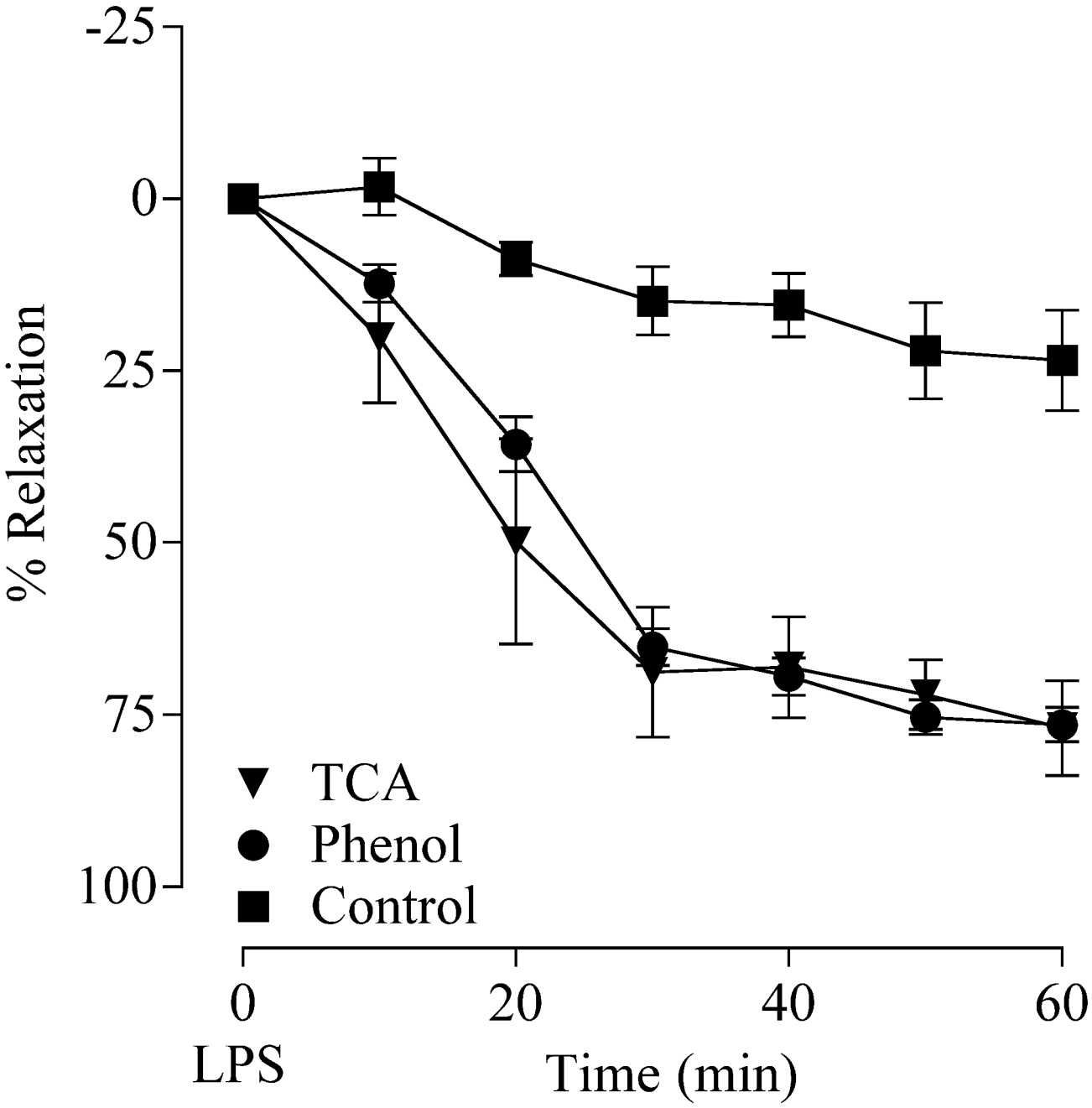
non-specific COX-inhibitor indomethacin (3 μM; n = 4) and the LPS antagonist polymixin B (10 $\mu g.ml^{-1}$, n = 4), (B) the specific COX-1 and COX-2 inhibitors SC560 (0.3 μM, n = 5) and SC236 (1 μM, n = 5) and their control (n = 8) and (C) the mixed DP/EP1/EP2 antagonist AH6809 (3 μM) and the specific EP4 antagonist L-161982 (1 μM) either alone (n = 4) or combined (n = 6) on the response of mouse isolated trachea to LPS. Control n =8.

Figure 5. (A) Effect of the p38 MAPK inhibitor SB203580 (10 μM, n = 4) on the response to LPS in the mouse isolated trachea. Control n = 8. (B) Effect of the NF-κB antagonist MG-132 (10 μM, n = 6), its vehicle DMSO (30 μL, n = 8) and the $cPLA_2$ inhibitor $AACOCF_3$ (10 μM, n = 8) on the response to LPS of mouse isolated trachea. For the control curve for $AACOCF_3$, n = 7. * $P < 0.05$ from respective control value at each time point.

Figure 6. Effect of the transcription inhibitor actinomycin D (2 μM, n = 4) and the translation inhibitor cycloheximide (10 μM, n = 4) on the response to LPS in mouse isolated trachea when added 40 min before LPS. Control n = 4.

Figure 7. Effect of the selective COX-1 and COX-2 inhibitors SC560 (0.3 μM) and SC236 (1 μM) respectively, either alone ($n \geq 3$), or combined ($n \geq 3$) on concentration-response curves for (A) substance P (n = 14), (B) SLIGRL (n = 16) or (C) combined substance P and SLIGRL (n = 3).

Figure 1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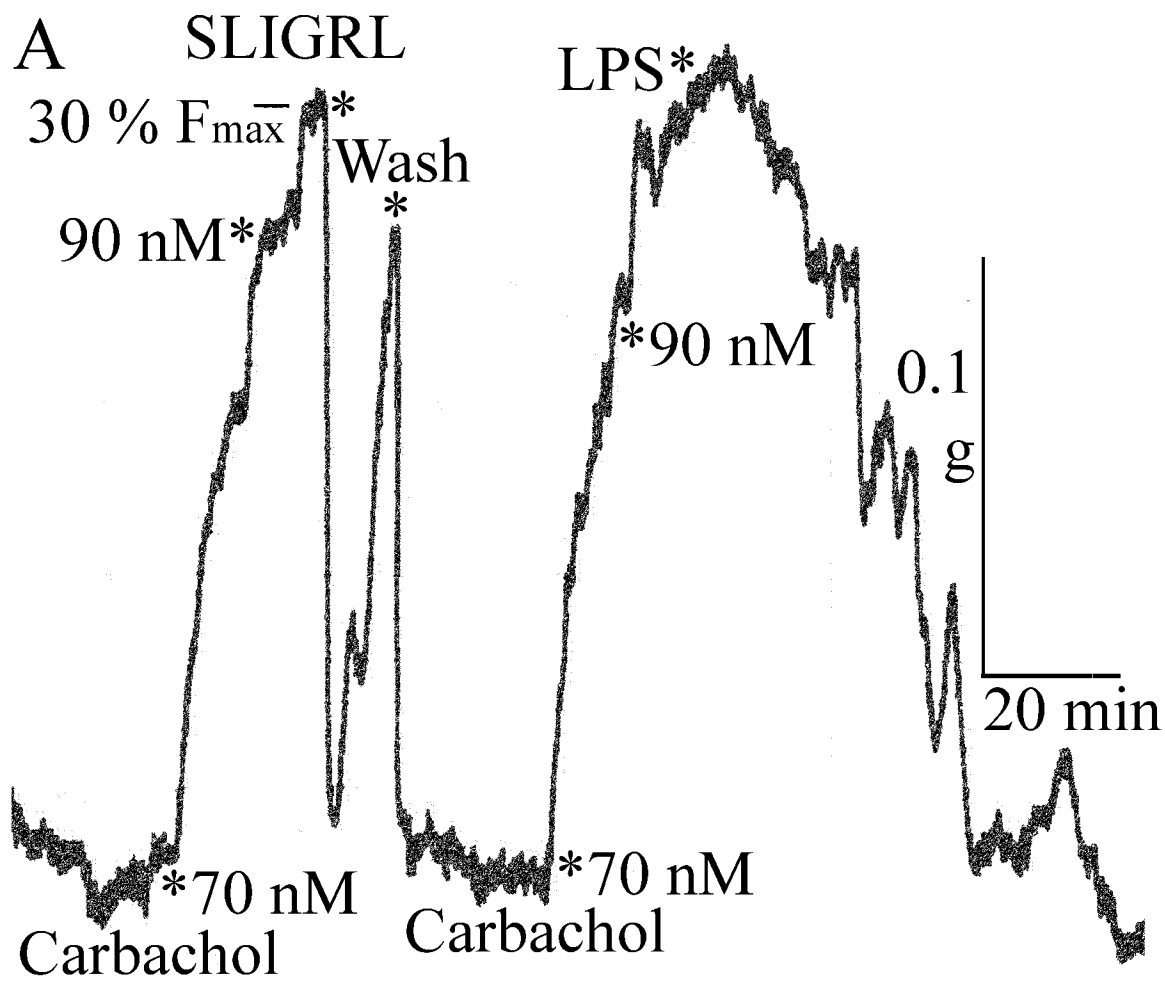
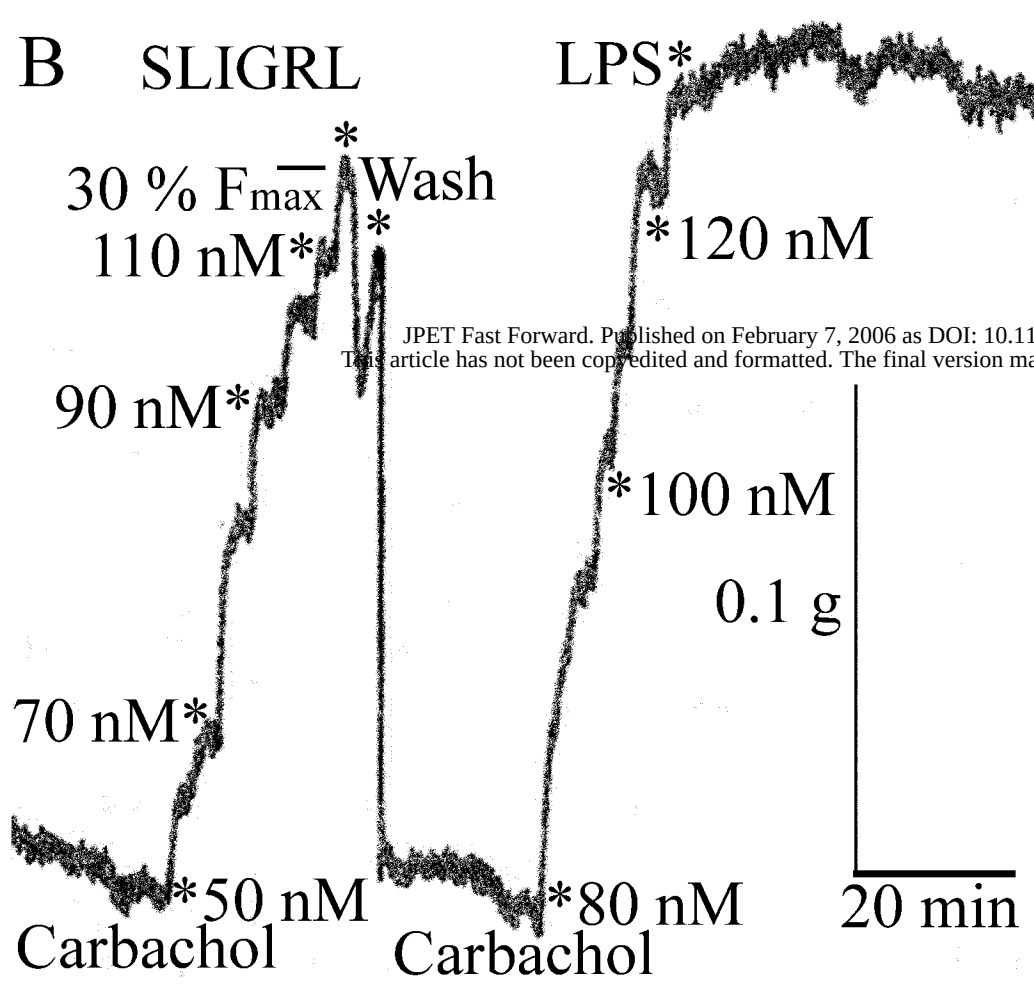
75
100
% Relaxation
TCA
Phenol
Control
0
20
40
60
LPS
Time (min)

Figure 2





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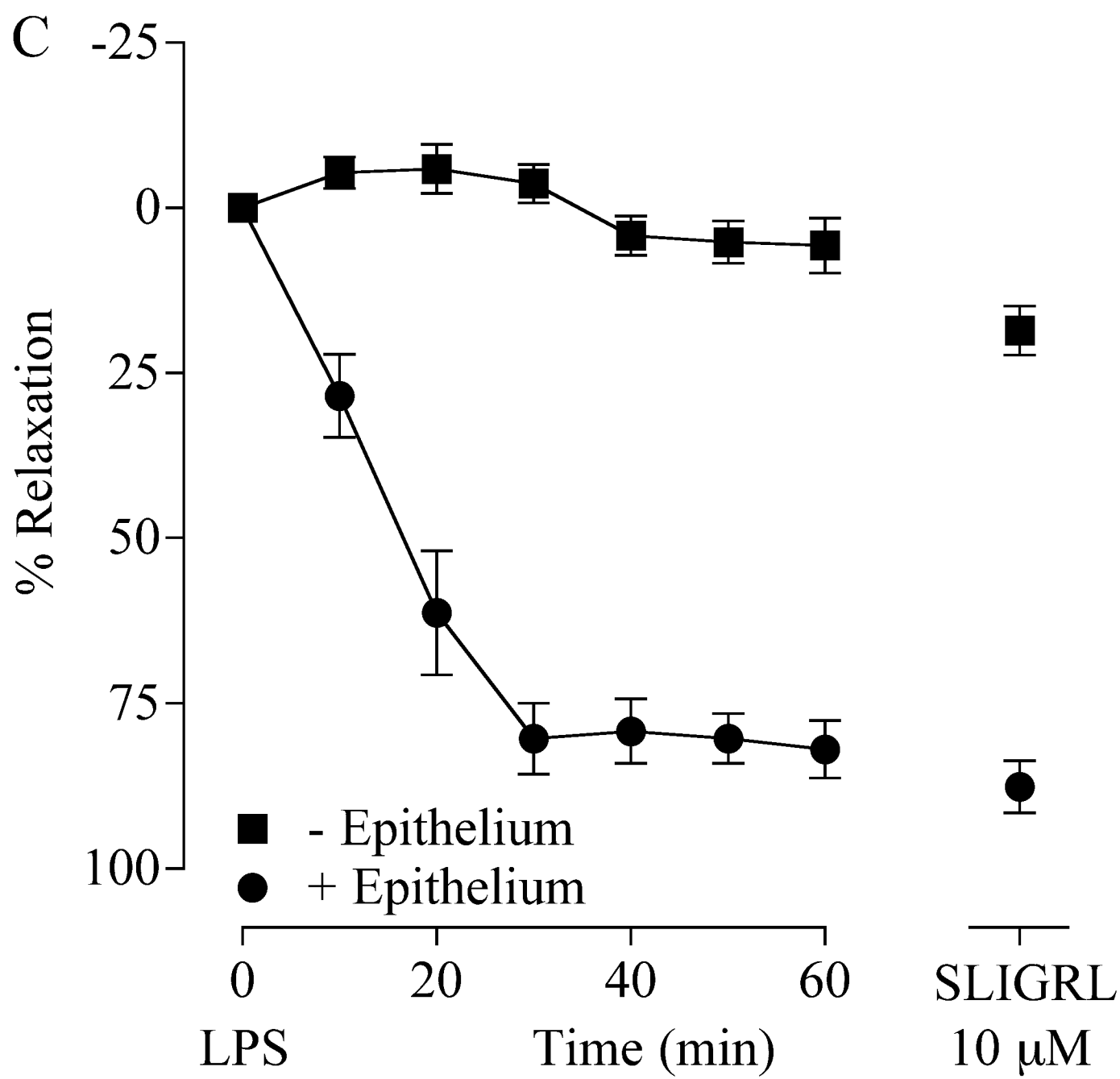

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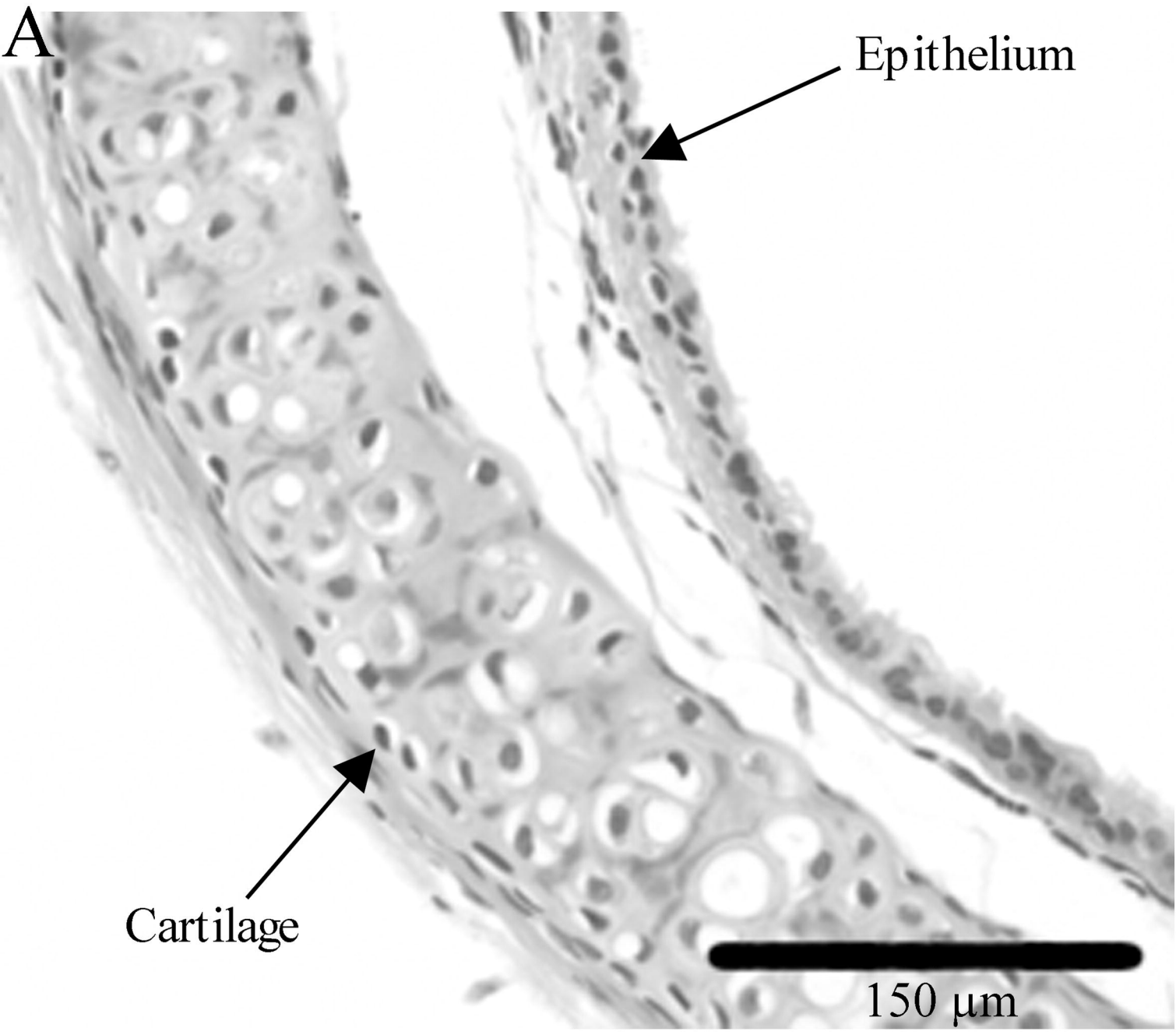

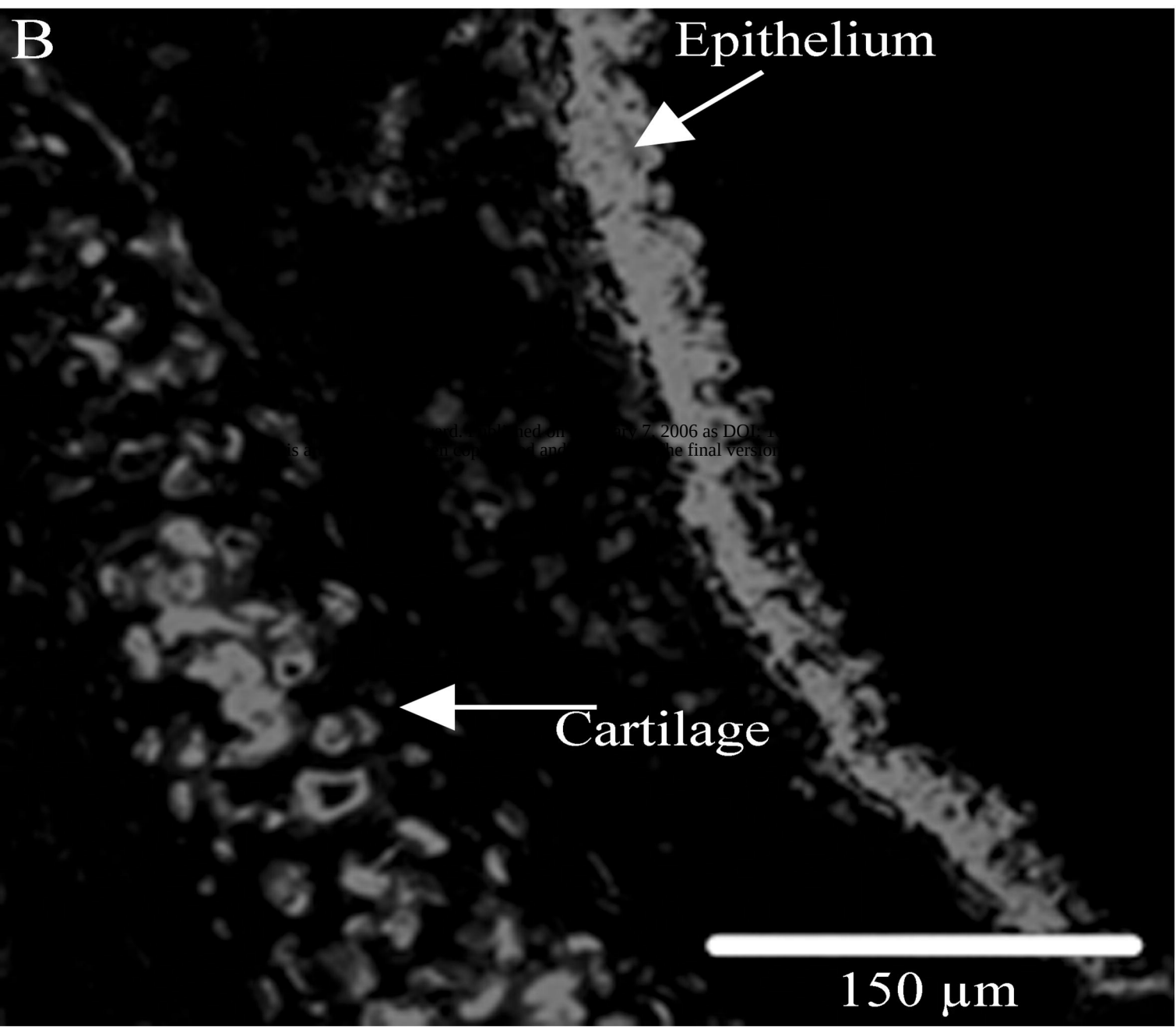

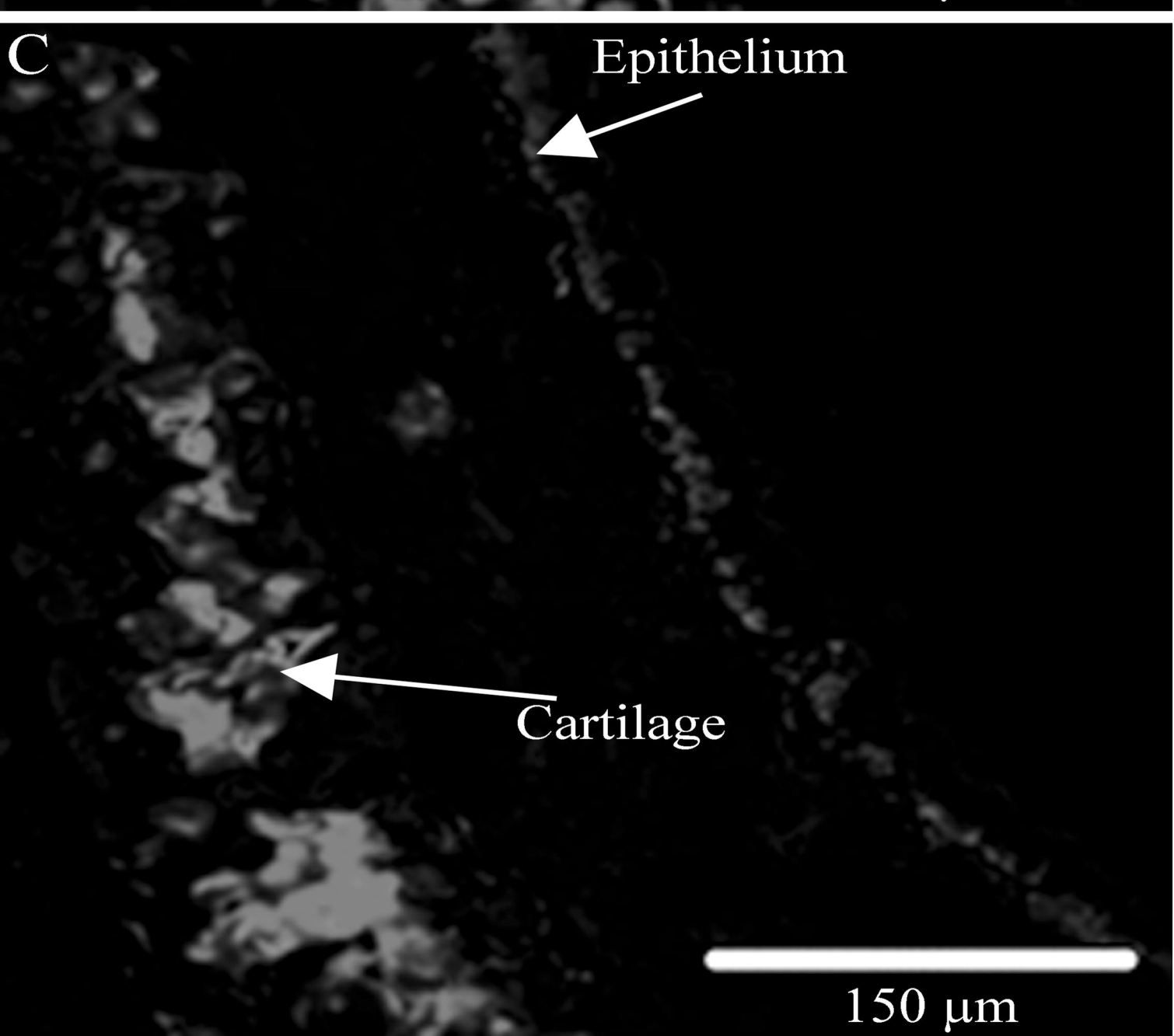

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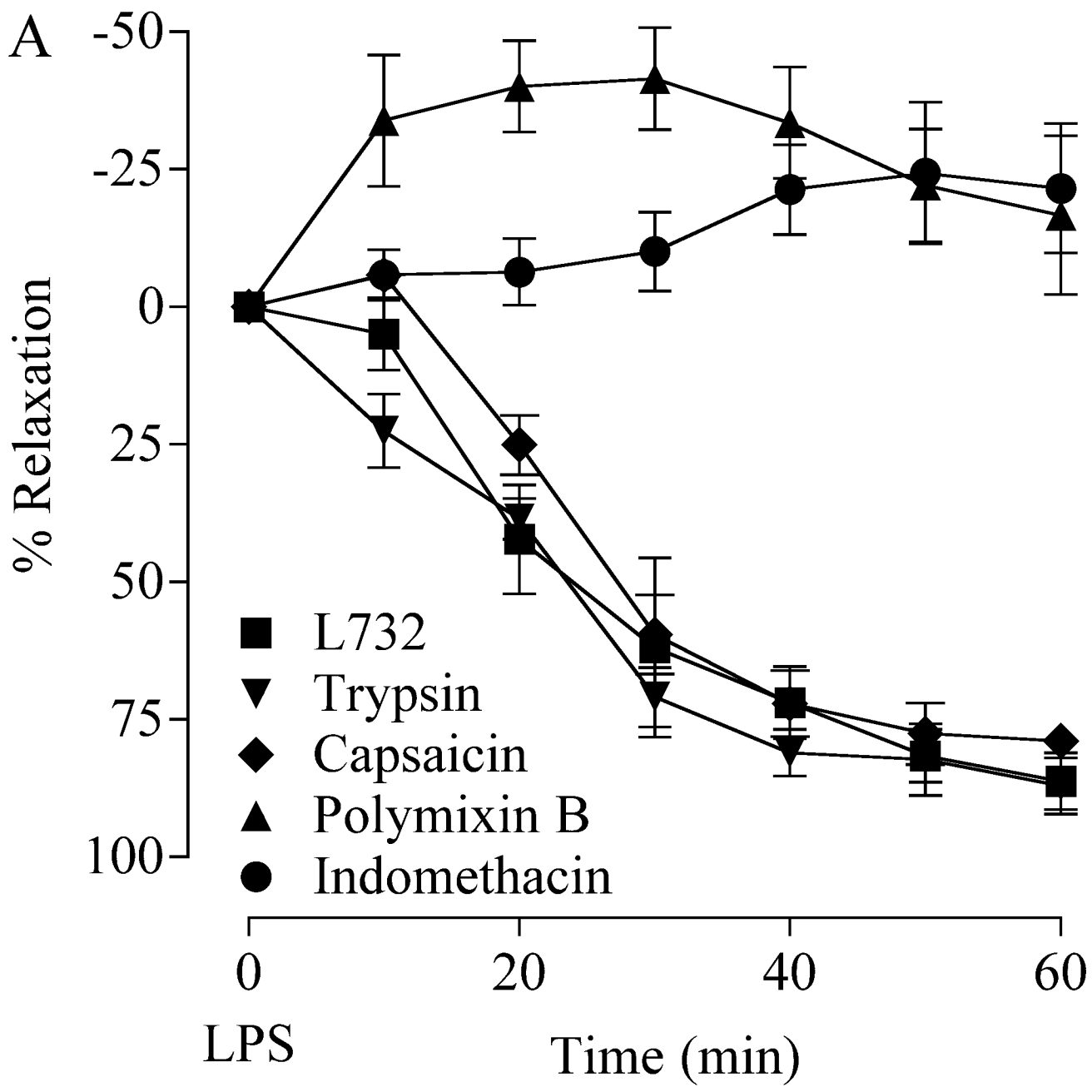

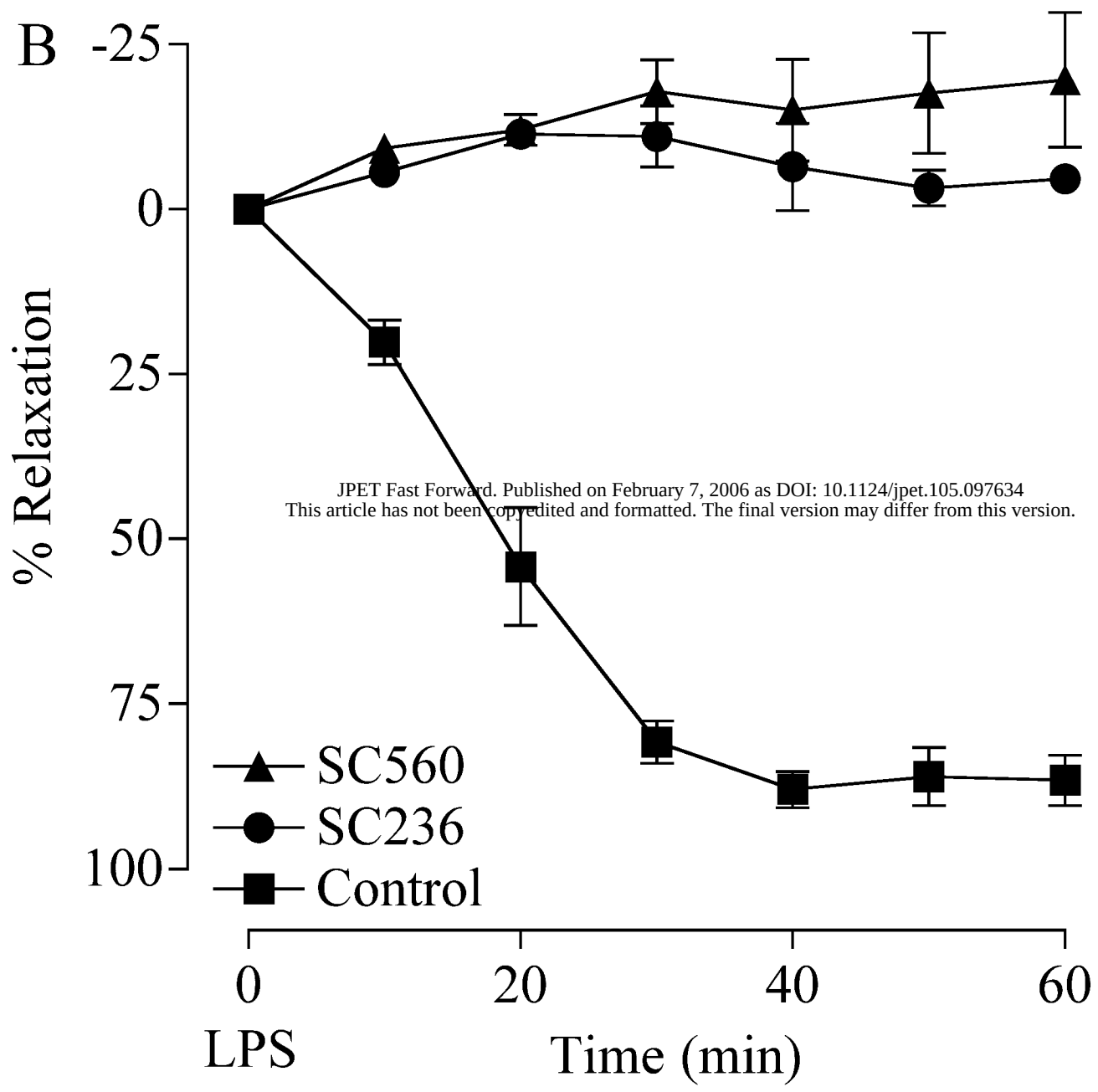

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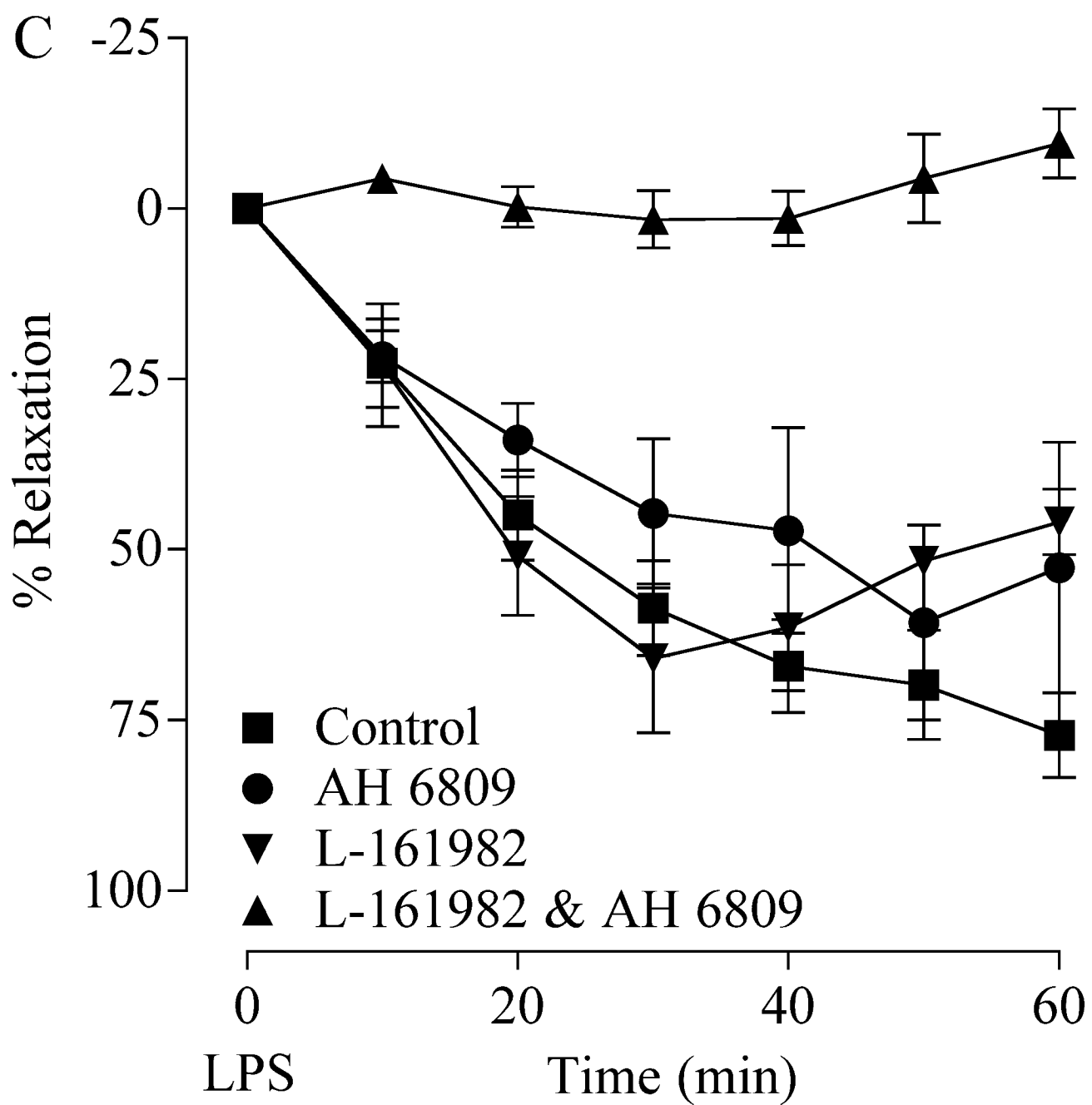
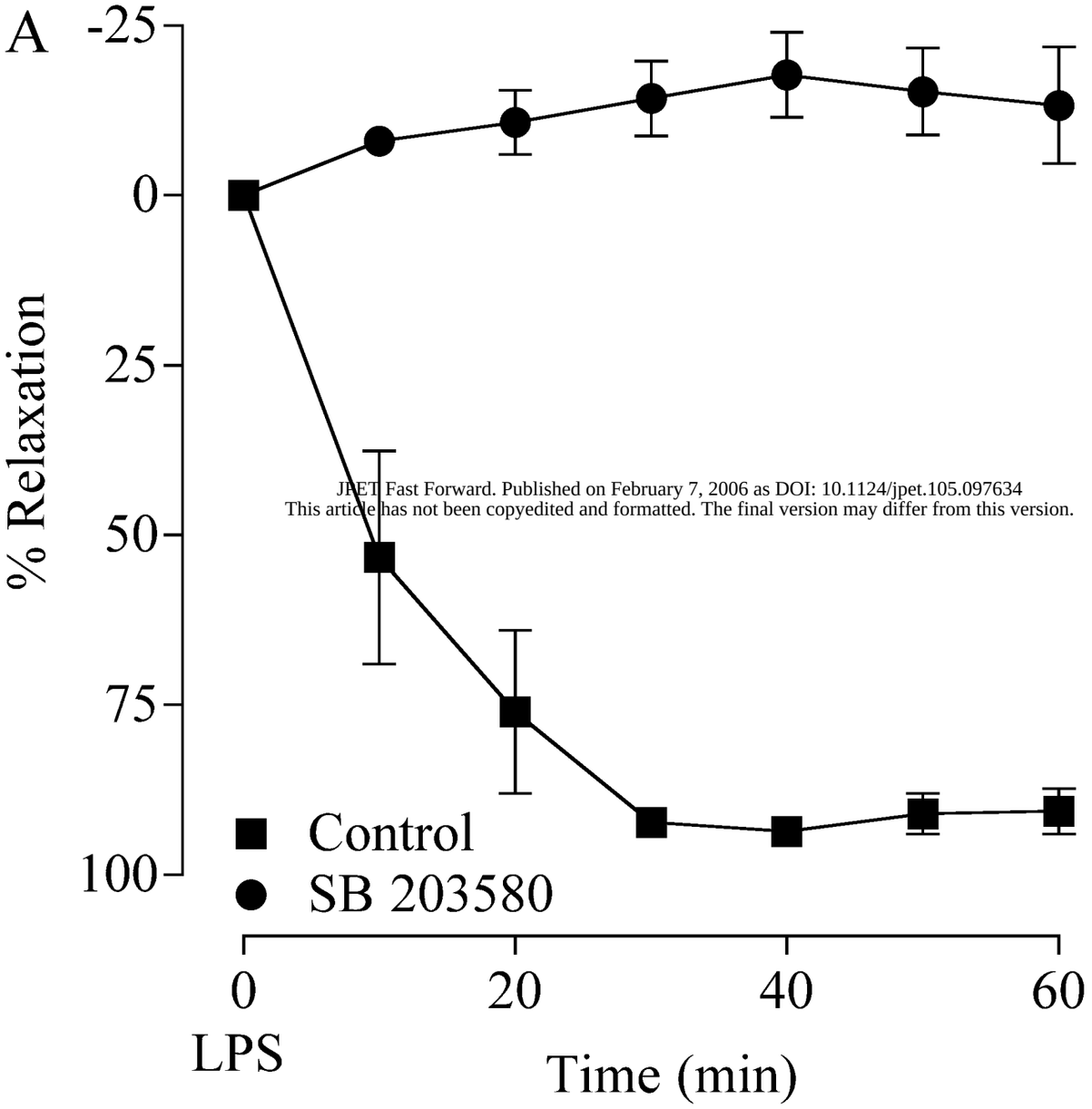
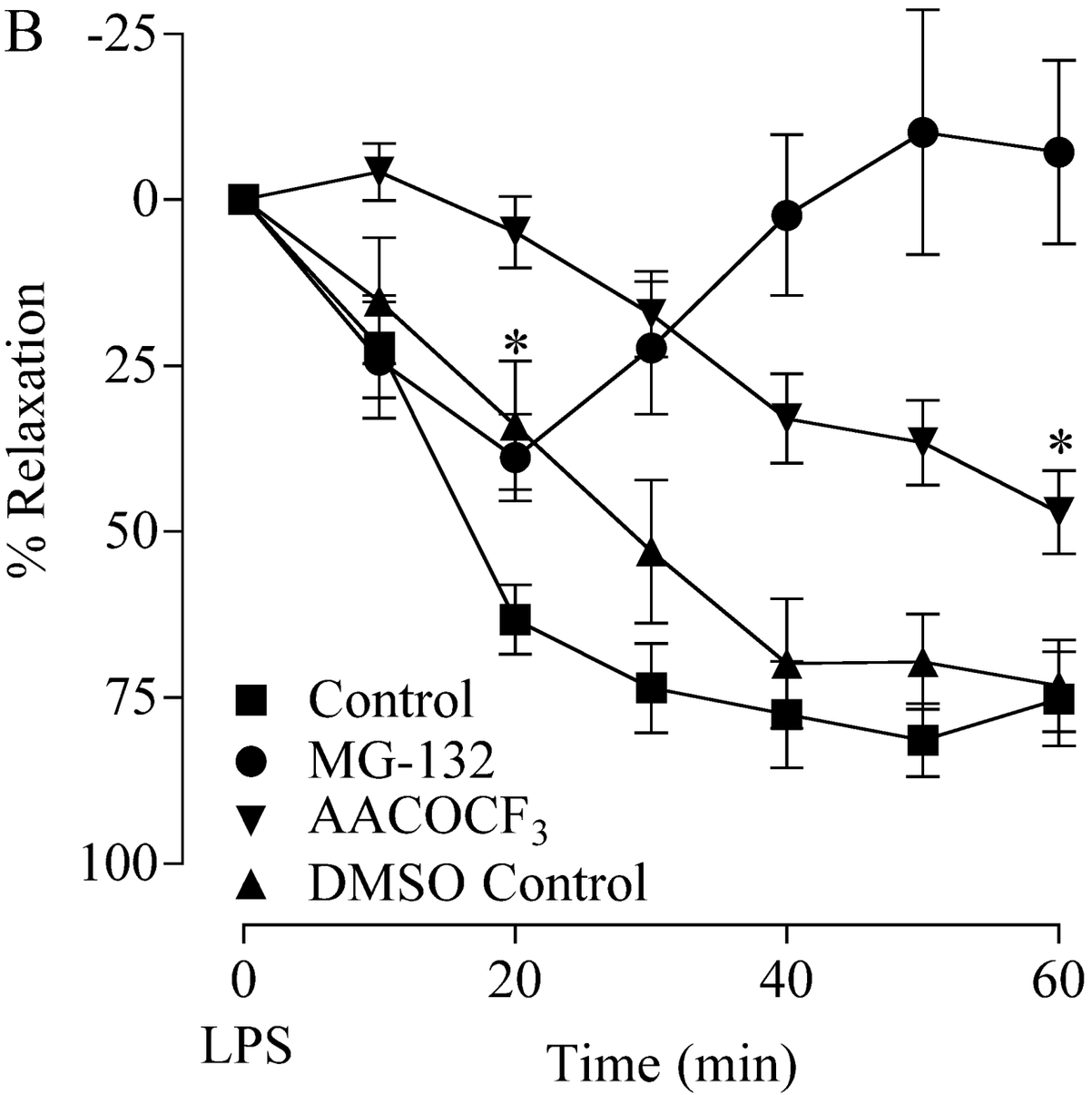


Figure 5





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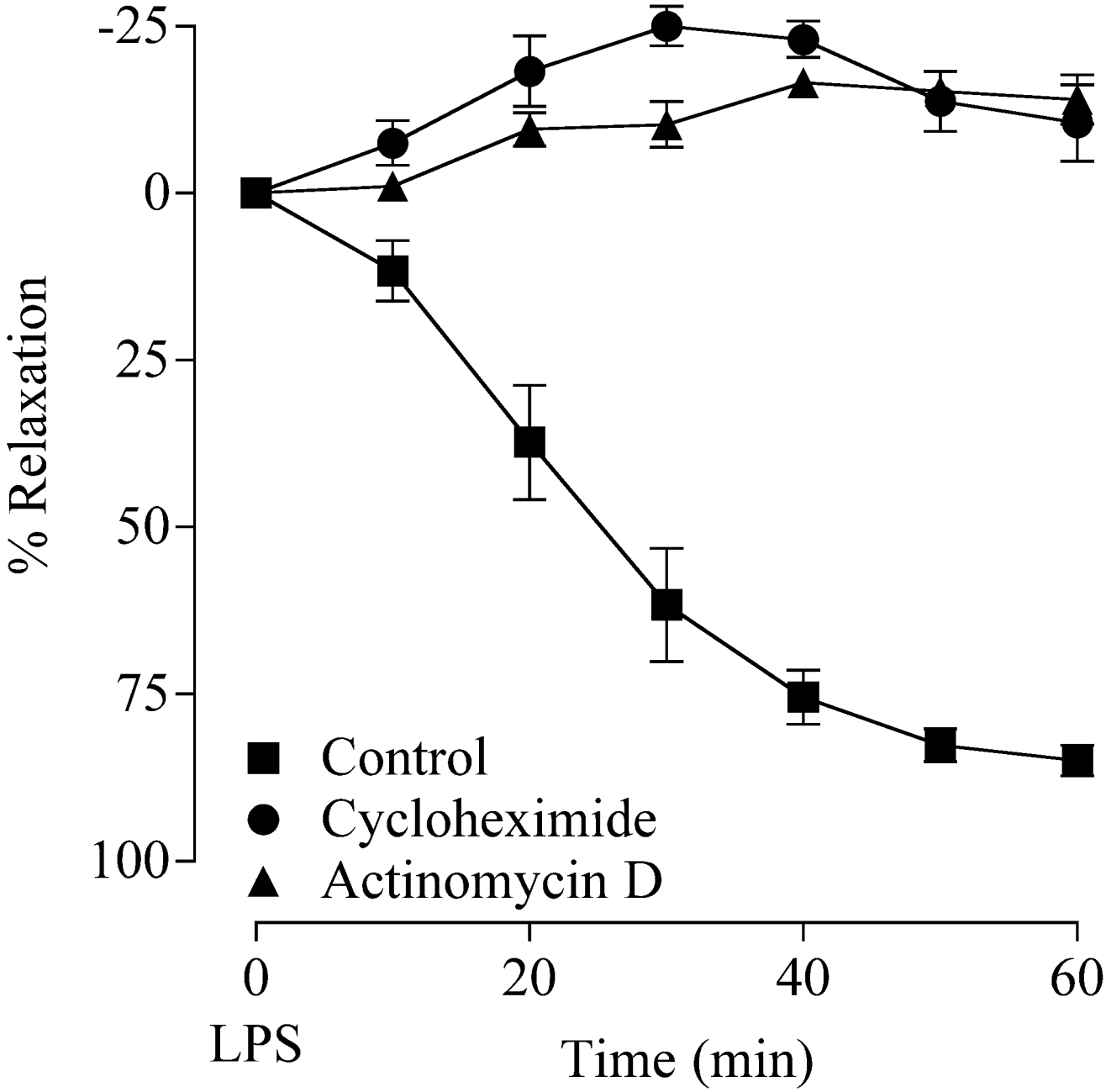
-25
0
25
50
75
100
% Relaxation
Control
Cycloheximide
Actinomycin D
0
20
40
60
LPS
Time (min)

Figure 7