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Pharmacology Biochemistry & Behavior, Vol. 55, No. 2, pp. 179–184, 1996
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0091-3057/96 $15.00 + .00

PII S0091-3057(96)00051-2

Inhibition of Nitric Oxide Synthase by Isothioureas: Cardiovascular and Antinociceptive Effects

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Received 5 October 1995; Revised 3 February 1996; Accepted 3 February 1996

HANDY, R. L. C., P. WALLACE AND P. K. MOORE. *Inhibition of nitric oxide synthase by isothioureas: Cardiovascular and antinociceptive effects.* PHARMACOL BIOCHEM BEHAV **55**(2) 179–184, 1996.—A range of substituted isothiourea compounds including S-isopropylisothiourea (IPTU), S-methylisothiourea (SMT), S-ethylisothiourea (ETU), N-pentylisothiourea (PTU), S-(2 aminoethyl)isothiourea (AETU), and S-acetamidoisothiourea (AATU) inhibit mouse spinal cord/cerebellar neuronal nitric oxide synthase (nNOS) and bovine aortic endothelial cell eNOS in vitro. IP administration of isothioureas increased mean arterial blood pressure of the urethane-anaesthetised mouse (rank order of effect: IPTU > ETU > SMT > AETU). PTU and AATU were without vasopressor activity. IPTU (50 mg/kg, IP) inhibited late phase formalin-induced hindpaw licking behaviour in the mouse while SMT (50 mg/kg, IP) was without effect. Neither compound influenced the formalin-induced increase in hindpaw weight reflecting a lack of significant peripheral antioedema effect in this model. IPTU (50 mg/kg, IP) but not SMT (50 mg/kg, IP) inhibited mouse spinal cord and cerebellar NOS activity measured ex vivo in animals killed 45 min after injection. The present study confirms the potent NOS inhibitory effect of selected substituted isothioureas in vitro. Little or no isoform selectivity (i.e., nNOS vs. eNOS) was apparent. The potent vasopressor effect of isothioureas indicates that these compounds may be of limited use as tools to study the role of nitric oxide in pain perception. **Copyright © 1996 Elsevier Science Inc.**

Isothiourea Methylisothiourea Isopropylisothiourea Nitric oxide synthase Blood pressure Antinociception

NITRIC oxide synthase (NOS), which is responsible for the formation of nitric oxide (NO) from L-arginine, exists in mammalian cells as three structurally distinct isoforms namely endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (12,13). NO has been the focus of intense study because it is believed to play an important role in a wide range of physiological functions (1,6,9). Recent research in this and other laboratories has centred upon the part played by NO in the mechanism of pain perception [reviewed in (8)]. Thus, we have previously reported that parenteral administration of inhibitors of NOS activity including L-N^G nitro arginine methyl ester [L-NAME; (10)] and 7-nitro indazole [7-NI; (11)] results in potent antinociceptive activity in the mouse assessed either as inhibition of formalin-induced hindpaw licking behaviour or as inhibition of acetic acid-induced abdominal constrictions. Recently, a number of substituted isothiourea compounds have been reported to inhibit NOS activity in vitro (3,14) and to increase blood pressure of the anaesthetised rat in vivo (14). To the best of our knowledge, neither the antinociceptive activity of isothiourea compounds nor their ability to inhibit spinal cord nNOS activity either in vitro or ex vivo has been reported in the literature. Accordingly, we have now evaluated a range of substituted isothiourea compounds as inhibitors of bovine aortic eNOS and mouse spinal cord and cerebellar nNOS activity in vitro as well as for their ability to increase blood pressure of the anaesthetised mouse and to inhibit formalin-induced hindpaw licking behaviour in the same species.

METHOD

Preparation and Assay of Mouse Spinal Cord/Cerebellar and Bovine Endothelial Cell NOS

The experimental procedures employed have been described in detail elsewhere (2,10,11). Briefly, mice (male,

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LACA, 25–35 g) were killed by cervical dislocation. Routinely, tissue from four to six mice was pooled for these studies. Spinal cords (combined thoracic and lumbar regions) and cerebella were rapidly dissected, weighed, and homogenised in an Ultra-Turrax (type 18/2N) homogeniser in 5 (spinal cord) or 10 (cerebellum) volumes of 20 mM Tris HCl buffer (pH 7.4) containing 2 mM EDTA. Tissues were snap frozen in liquid nitrogen and stored at −70°C until used. Homogenates were centrifuged (10,000 × g) for 15 min at 4°C and the crude supernatant used for NOS assay as described below.

Bovine aortae were obtained from a local slaughterhouse and transferred to the laboratory on ice within 3 h of death. Vascular endothelial cells were removed by careful scraping of the intimal surface with a scalpel blade. The crude cell suspension was washed twice with phosphate-buffered saline (pH 7.4), centrifuged (10,000 × g, 5 min, 4°C), and endothelial cells subsequently homogenised in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1 mM EDTA, 0.1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 1% (v/v) 2-β-mercaptoethanol with 25 passes of a glass Teflon Dounce homogeniser.

NOS activity was assayed by monitoring the conversion of [^{3}H] L-arginine to [^{3}H] citrulline. Incubations (15 min at 0°C followed by 15 min at 37°C) contained 25 μl enzyme supernatant, 0.5 μCi [^{3}H] L-arginine (concentration = 120 nM), 0.75 mM $CaCl_2$, 0.5 mM NADPH and 5 μl water or drug solution (final concentration, 0.1 μM–10 mM) in a total volume of 105 μl. The reaction was started by addition of radiolabel and transfer to 37°C. Assays were terminated by addition of 3 ml HEPES buffer (pH 5.5) containing 2 mM EDTA and incubates applied to 0.5 ml columns of Dowex AG50WX-8 (Na^+) form followed by 0.5 ml distilled water to remove unchanged [^{3}H] L-arginine. [^{3}H] citrulline was quantified by liquid scintillation spectroscopy of a 1 ml aliquot of the combined flowthrough. Protein concentration of tissue supernatants was measured by the Folin-phenol reaction (7).

In separate experiments mice were injected IP with test compounds or saline (0.9% w/v NaCl, 0.1 ml/10 g) and killed by cervical dislocation 45 min thereafter. Spinal cords and cerebella were removed, homogenised, and assayed for NOS activity as described above. Results are expressed as pmol citrulline/15 min/mg protein.

Measurement of Mouse Blood Pressure

Mice (male, LACA, 25–35 g) were anaesthetised with urethane (10 g/kg, IP) and a saline-filled cannula (type 0.63 mm diameter drawn out to a smaller gauge of approximate diameter 0.25 mm) inserted into the right carotid artery. Mean arterial blood pressure (MAP) was monitored continuously using a Druck pressure transducer connected to a MacLab 2E using a Macintosh Performa 475 microcomputer. Test drugs or saline (0.1 ml/10 g) were administered IP as a bolus and the effect on MAP determined over the following 45 min.

Assessment of Antinociceptive Activity

The antinociceptive activity of isothioureas was determined essentially as described elsewhere (5). Mice (male, LACA, 25–35 g) were acclimitised to the test room (ambient temperature, 23°C) for a minimum of 1 h prior to the start of the experiment. All experiments were performed in the period 1300–1700 h. Test drugs or saline (0.1 ml/10 g) were administered IP 15 min prior to subplantar injection of formalin (5% v/v, 10 μl) into the right hindpaw. Thereafter, animals were immediately transferred to transparent Plexiglas observation chambers and hindpaw licking time monitored in the periods 0–5 min (early phase) and 15–30 min (late phase) following formalin injection. Results are expressed as hindpaw licking time in seconds. Animals were carefully observed throughout the test period for alternative drug-induced behavioural changes, for example, alterations in locomotor or grooming activity, changes in gait and/or muscle ataxia. At the end of the experiment animals were killed and the injected hindpaw removed and weighed to provide an index of formalin-induced hindpaw oedema formation.

Drugs and Chemicals

S-methylisothiourea (SMT) was purchased from Aldrich Ltd. S-Isopropylisothiourea (IPTU), N-pentylisothiourea (PTU), S-ethylisothiourea (ETU), S-(2 aminoethyl)isothiourea (AETU), and acetamidoisothiourea (AATU) were provided by Dr. G. J. Southan and Dr. C. Thiemermann (William Harvey Research Institute, St. Bartholomew's Hospital, London, UK). Radiolabelled [^{3}H] L-arginine (sp. act. 62 Ci $mmol^{-1}$) was obtained from Amersham Ltd (UK). Dowex AG50WX-8 H^+ form (Sigma Ltd., UK) was converted into the Na^+ form by soaking for 2 h in 2 M NaOH followed by repeated washing in water until neutrality. All other drugs and chemicals were purchased from Sigma Ltd. (UK). Drug stocks were prepared fresh on the morning of each experiment. Isothiourea compounds were dissolved in water (in vitro experiments) or saline (in vivo experiments).

Statistical Analysis

Results show mean ± SE mean with number of observation indicated in parenthesis. Statistical significance of differences between groups was determined using unpaired Student's *t*-test or where multiple comparisons were undertaken by ANOVA with posthoc Dunnett's test. A probability (p) value not exceeding 0.05 was taken to indicate a significant difference.

RESULTS

Effect of Isothiourea Compounds on NOS In Vitro

Control NOS enzyme activity in the absence of added test drug was 3.38 ± 0.59, 12.76 ± 2.78, and 1.76 ± 0.41 pmol citrulline/15 min/mg protein (n = 6) for homogenates prepared from mouse spinal cord, cerebellum, and bovine aortic endothelial cells, respectively. All six isothiourea compounds studied caused concentration related inhibition of NOS enzyme activity in vitro (Fig. 1). The concentrations (μM) of each compound required to cause 50% inhibition (i.e., IC_{50}) of mouse spinal cord, cerebellar, and bovine aortic endothelial cell NOS, respectively, were as follows: ETU (2.06, 0.45, 0.28), IPTU (0.66, 0.75, 0.29), SMT (2.56, 2.14, 0.28), AETU (31.46, 18.67, 6.27), PTU (78.71, 89.08, 60.49), and AATU (1252, 2018, 73.9).

Effect of SMT and IPTU on Mouse NOS Activity Determined Ex Vivo

Administration of IPTU (50 mg/kg, IP) inhibited both spinal cord and cerebellar NOS measured ex vivo in animals killed 45 min thereafter by 53.4 ± 3.1% and 79.7 ± 0.3% (n = 4), respectively. In similar experiments, SMT (50 mg/kg, IP) failed to influence either mouse spinal cord or cerebellar NOS under these conditions (Fig. 2).

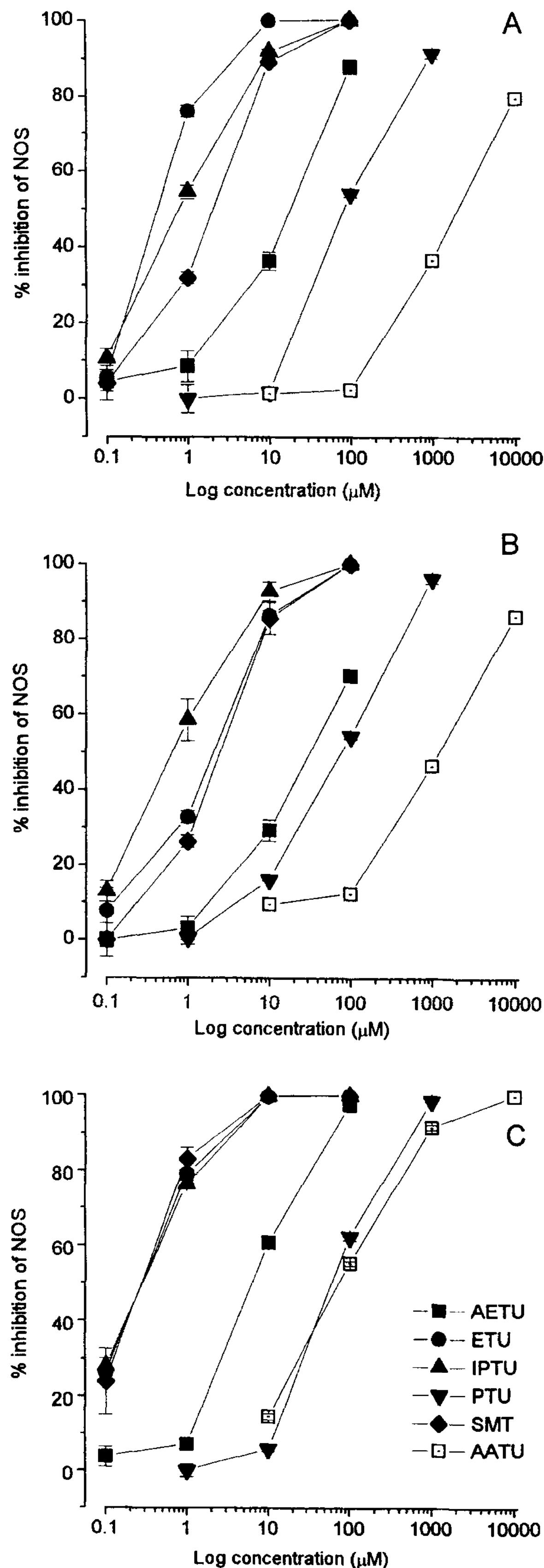


FIG. 1. Inhibition by isothiourea compounds of mouse cerebellar (A), mouse spinal cord (B) and bovine aortic endothelial cell (C) NOS activity. NOS activity was determined as the conversion of [^{3}H] L-arginine to [^{3}H] citrulline. Results show % inhibition of enzyme activity and are mean ± SE mean, $n = 6$.

Effect of Isothiourea Compounds on Mouse Blood Pressure

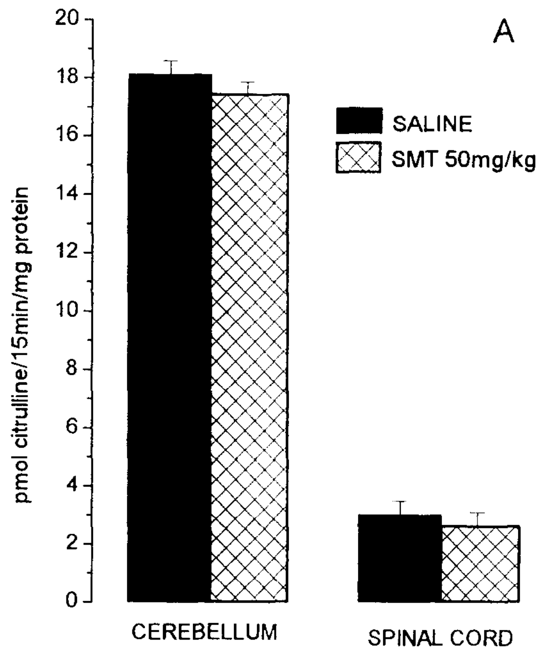
The resting MAP of mice used in this study was 50.7 ± 1.5 mmHg ($n = 42$). Administration of saline (0.1 ml/10 g, IP) resulted in a gradual but statistically nonsignificant ($p > 0.05$) decrease in MAP (42.1 ± 3.8 mmHg, $n = 5$) at 45 min postinjection (Fig. 2). Similarly, SMT (0.1 mg/kg, IP) also caused a small time-dependent decrease in MAP that was not significantly different at 45 min postinjection compared with that observed in saline-injected animals (41.8 ± 2.9 mmHg, $n = 4$, $p > 0.05$). Higher doses of SMT (1,10 and 50 mg/kg, IP) produced graded increases in MAP that were rapid in onset (usually within 2 min of injection) and plateaued within 15 min. Of the other isothiourea compounds tested (all 50 mg/kg, IP) ETU, IPTU, and AETU also increased MAP, while PTU and AATU were almost inactive (Fig. 3).

Effect of SMT and IPTU Formalin-Induced Hindpaw Licking Behaviour and Hindpaw Weight Gain

Early and late phase hindpaw licking times in control (saline-injected) animals used in this study were 75.6 ± 3.5 s and 105.1 ± 6.2 s, respectively ($n = 54$). Administration of SMT (50 mg/kg, IP) failed to influence either the early (87.7 ± 6.1 s, cf. 92.7 ± 7.0 s in vehicle-injected animals, $n = 12$, $p > 0.05$) or late phase (72.7 ± 13.0 s, cf. 89.0 ± 12.4 s in vehicle-injected animals, $n = 12$, $p > 0.05$) hindpaw licking behaviour. In contrast, IPTU (50 mg/kg, IP) significantly inhibited the late phase (but not the early phase) hindpaw licking response. Lower doses of IPTU (10 and 20 mg/kg, IP) were inactive (Fig. 4). Subjective observation of animals during the test period suggested a reduction in locomotor activity and normal grooming activity in animals injected with IPTU (50 mg/kg, IP). SMT (50 mg/kg, IP) and lower doses of IPTU (10–20 mg/kg, IP) did not appear to affect animal behaviour. Subplantar formalin injection in all animals studied resulted in an increase in hindpaw weight of 63.7 ± 2.9 mg ($n = 54$). This increase was not influenced by pretreatment with either SMT (50 mg/kg; 72.6 ± 4.9 mg, $n = 12$, cf. 62.9 ± 3.9 mg in vehicle-injected animals, $n = 12$, $p > 0.05$) or IPTU (50 mg/kg; 71.2 ± 4.5 mg, $n = 12$, cf. 64.7 ± 1.9 mg, $n = 12$, $p > 0.05$).

DISCUSSION

The rank order of potency of isothiourea compounds as inhibitors of mouse spinal cord/cerebellar nNOS and bovine aortic endothelial cell eNOS isoforms was IPTU = ETU = SMT > AETU > PTU > AATU. Although each of the compounds studied proved to be somewhat more potent inhibitors of eNOS than nNOS with IC_{50} ratios (spinal cord nNOS:eNOS) ranging from 1.3 (PTU) to 16.9 (AATU), the present results do not indicate substantial selectivity amongst these compounds for inhibition of nNOS vis-à-vis eNOS, at least in vitro. Essentially, similar IC_{50} values have been reported for these compounds against both bovine aortic endothelial cell eNOS and J774 macrophage iNOS (14). Interestingly, Garvey and colleagues (3) have reported that SMT, IPTU, and a range of other isothiourea compounds inhibit all three purified human NOS isoforms with K_i values in the low nanomolar range. The reason for the discrepancy in the



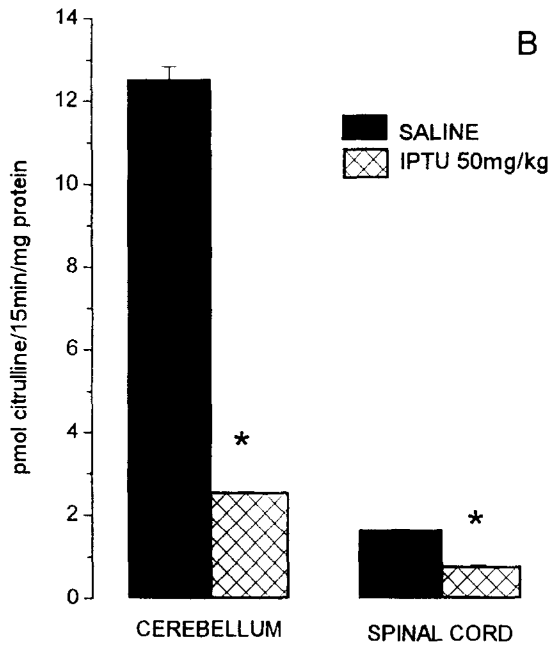
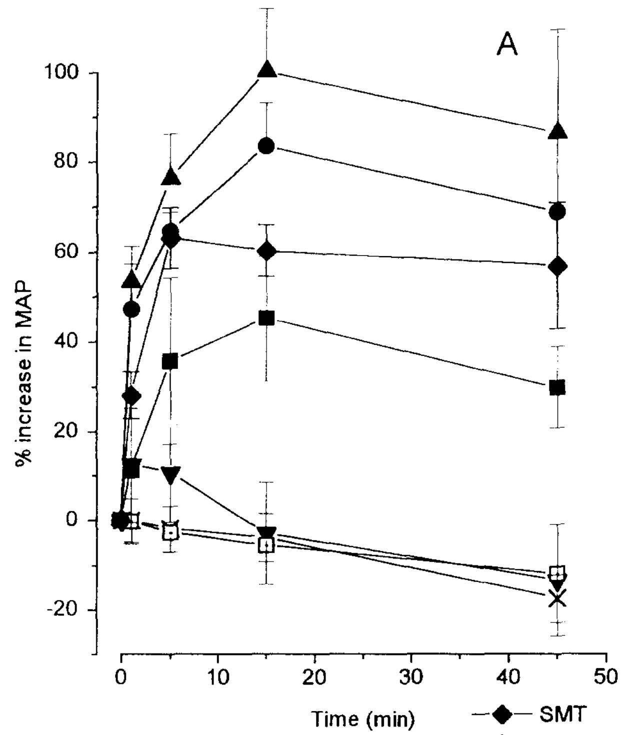


FIG. 2. Effect of (A) SMT (50 mg/kg) and (B) IPTU (50 mg/kg) on cerebellar and spinal cord NOS enzyme activity measured 45 min after IP drug administration. NOS activity was determined as the conversion of [^{3}H] L-arginine to [^{3}H] citrulline and results indicate pmol citrulline produced/15 min/mg protein, $n = 4$, $*p < 0.01$.



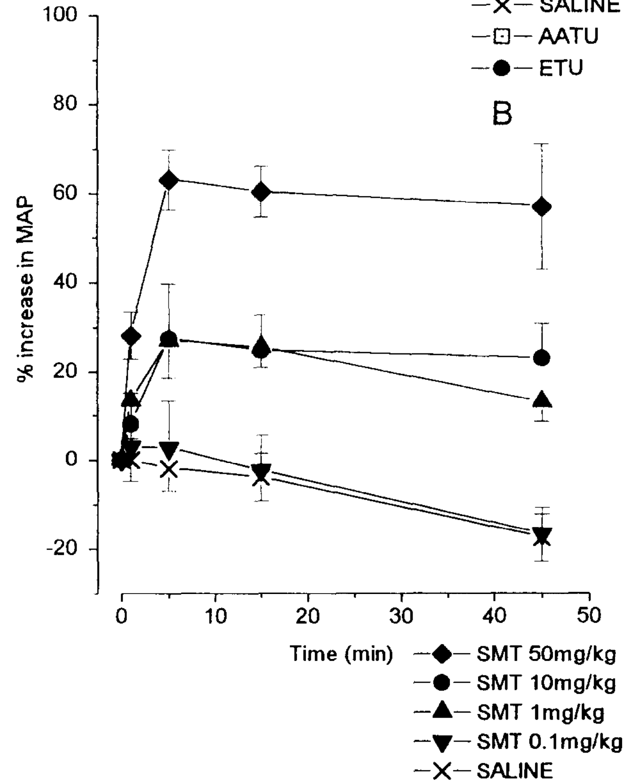


FIG. 3. (A) Time dependent effect of isothiourea compounds on MAP of urethane anaesthetised mice. (B) Dose-related increase in MAP of urethane anaesthetised mice following administration of SMT. Results show % change in MAP and are mean ± SE mean, $n = 4$–7.

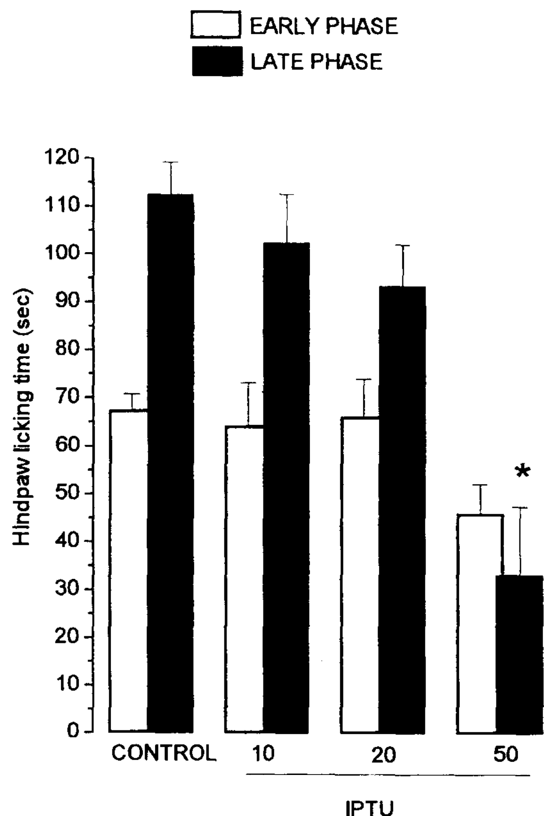


FIG. 4. Effect of IPTU (10–50 mg/kg, IP) on formalin-induced hindpaw licking behaviour in the mouse. Results are expressed as hindpaw licking time in seconds and are mean ± SE mean, $n = 12$, $*p < 0.01$.

reported potency of isothioureas as NOS inhibitors between these studies is not clear. Although the ability of isothioureas to inhibit NOS may be subject to species differences, the observation that greatest inhibition was observed in experiments utilising purified NOS isoforms (3) raises the possibility that the lower potency of isothioureas in the present study reflects either catabolism by cytoplasmic enzymes or perhaps binding to cellular components present in the crude tissue homogenates employed.

The ability of SMT and related isothiourea compounds to increase MAP in the anaesthetised mouse (secondary to inhibition of vascular eNOS) and to inhibit formalin-induced hindpaw licking behaviour (secondary to inhibition of nNOS) was also evaluated in vivo. Thus, SMT administered IP to the anaesthetised mouse produced a dose-related increase in MAP that was both rapid in onset and persisted throughout the 45 min of the experiment. On a weight-for-weight basis, SMT was approximately equipotent with L-NAME as a vasopressor in this species (10). Comparison of the vasopressor effect of a single dose of all six isothiourea compounds studied revealed a rank order of effect (IPTU > ETU > SMT > AETU > PTU, and AATU), which was similar to that for inhibition of bovine aortic endothelial cell NOS in vitro. Thus, the present data strongly suggests that inhibition of vascular endothelial NOS accounts for the ability of SMT and related isothiourea compounds to increase MAP in the anaesthetised mouse.

Like L-NAME (10) and 7-NI (11), administration of the highest dose of IPTU used in this study resulted in antinociception in the mouse as determined by the inhibition of late phase formalin-induced hindpaw licking behaviour. However, on a weight-for-weight basis, IPTU was considerably less potent than either L-NAME or 7-NI [ED_{50} values approximately 20 mg/kg, IP; (10,11)] Neither IPTU nor SMT reduced the albeit modest increase in hindpaw weight (indicative of oedema formation) that follows subplantar formalin injection in the mouse, suggesting that an antiinflammatory effect is unlikely to underlie the IPTU-induced antinociception at least in this experimental model. The finding that IP administration of IPTU significantly reduced mouse spinal cord NOS enzyme activity determined ex vivo supports the possibility that the antinociceptive effect of this compound is secondary to NOS inhibition within the spinal cord.

Unlike IPTU, SMT was without effect on either the early or late phases of formalin-induced hindpaw licking behaviour and also failed to inhibit mouse spinal cord NOS enzyme activity determined ex vivo. These results are somewhat surprising in view of the structural similarity of SMT and IPTU and their similar potency as inhibitors of mouse spinal cord nNOS in vitro. The inability of SMT to inhibit spinal cord NOS after IP administration may suggest that SMT fails to gain access to spinal cord NOS-containing neurones following IP administration. The potent and dose-related vasopressor effect of SMT in the anaesthetised mouse would argue against extensive plasma binding or enzymatic inactivation of this compound in the periphery. Thus, the lack of antinociceptive effect of SMT may result from limited access to or perhaps accelerated catabolism of this compound in spinal cord NOS-containing neurones. Clearly, further information concerning blood and tissue concentrations of SMT following parenteral administration in the mouse are required.

In conclusion, the present data confirms the ability of a range of substituted isothiourea compounds to inhibit eNOS enzyme activity in vitro and in vivo (as determined by increase in MAP of the anaesthetised mouse). No substantial isoform selectivity (eNOS vs. nNOS) was demonstrated in vitro using these compounds. Although a high dose of IPTU (but not SMT) exhibits antinociceptive activity in the mouse and inhibits spinal cord nNOS activity ex vivo, the pronounced vasopressor activity of this class of NOS inhibitors coupled with their reported toxicity following parenteral administration in experimental animals (4) renders it unlikely that isothioureas will prove of value as tools with which to evaluate the biological role of nitric oxide in pain perception.

ACKNOWLEDGEMENTS

We are grateful to the Arthritis and Rheumatism Research Council for financial support and to Dr. G. J. Southan and Dr. C. Thiemermann (William Harvey Research Institute, St. Bartholomew's Hospital, London) for the generous gift of isothiourea compounds.

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