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European Journal of Pharmacology 436 (2002) 53–56

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Short communication

Microglial derived nitric oxide decreases serotonin content in rat basophilic leukemia (RBL-2H3) cells

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Received 4 October 2001; received in revised form 10 December 2001; accepted 21 December 2001

Abstract

Nitric oxide (NO) and serotonin (5-hydroxytryptamine; 5-HT) are important neuromodulators that are involved in a myriad of biochemical reactions. In this work, we describe a novel model co-culture system to study the interactions between NO and 5-HT. NO derived from cytokine stimulated Bv2 microglial cells depleted 5-HT from RBL-2H3 cells. Reduction of 5-HT content by NO derived from the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) was concentration-dependent, independent of intracellular Ca^{2+} and inhibited by reduced glutathione (GSH). Collectively, these data indicate that this cell co-culture system is a viable model to study the mechanisms of interaction between nitrergic and serotonergic pathways.

Keywords: Nitric oxide (NO) synthase; 5-HT (5-hydroxytryptamine, serotonin); Neuromodulation

1. Introduction

Nitric oxide (NO) has been identified as a unique neurotransmitter with distinct biochemistry that enables it to act on diverse cellular targets. The biological actions of NO involve its interaction with reactive oxygen species, other free radicals, transition metals, thiols and molecular oxygen. The best characterized targets of NO are heme-containing proteins, particularly guanylyl cyclase (for review, see Lane and Gross, 1999; Yun et al., 1997).

Serotonin (5-hydroxytryptamine; 5-HT) is a major neuromodulator in central and peripheral nervous pathways. The effects of 5-HT are involved in such diverse and complex neural responses as digestion (Gershon, 1999), pain (Richardson, 1990; Roberts, 1989) and psychiatric disorders (Paul, 1999). Several lines of evidence indicate a high degree of interaction between NO and serotonergic transmission (Kuhn and Arthur, 1996; Prast and Philippu, 2001). A thorough understanding of the interaction between nitrergic and serotonergic pathways is of paramount importance. The current study describes a novel experimental model for use in studying the mechanisms of interaction between these neurotransmitter systems.

2. Methods

2.1. Cell culture

Rat basophilic leukemia cells (RBL-2H3; American Type Culture Collection, Manassas, VA) were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Gaithersburg, MD) supplemented with 10% bovine calf serum (Hyclone, Logan, UT), 100 units/ml penicillin (Gibco) and 100 μg/ml streptomycin (Gibco). Microglial cells (Bv2; a generous gift from Dr. M. McKinney, Mayo Clinic, Jacksonville, FL) were grown in DMEM supplemented with 10% fetal bovine serum (Gibco), 50 units/ml penicillin and 50 μg/ml streptomycin.

2.2. Effects of exogenous compounds on RBL-2H3 serotonin levels

Monolayers of RBL-2H3 cells in 24-well plates were washed three times with HEPES buffer (110 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 20 mM HEPES, 25 mM glucose and 60 mM sucrose; pH 7.4; 335-340 mosM), and incubated at 37 °C in a shaking water bath (65 rpm). *S*-nitroso-*N*-acetylpenicillamine (SNAP; Sigma, St.

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0014-2999/02/$ - see front matter
PII: S0014-2999(01)01615-6

Louis, MO) was added directly to each well and incubated with the cells for 30 min. In some experiments, addition of 1,2-bis(*o*-amino-5-fluorophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM; Calbiochem, La Jolla, CA) or reduced glutathione (GSH; Sigma) preceded the addition of SNAP. Cells were then lysed and an aliquot of the lysate was analyzed for 5-HT content by enzyme immunoassay according to the manufacturer's instructions (Beckman Coulter, Fullerton, CA).

2.3. Co-culture experiments

In experiments where RBL-2H3 cells were combined with Bv2 cells, RBL-2H3 cells were grown to confluence on Transwell inserts (0.4 μm pore; Corning Life Sciences, Acton, MA). This system allowed for the co-incubation of RBL-2H3 cells with Bv2 cells with intercellular communication only via diffusible signals. The cells were transferred to a 24-well plate that contained monolayers of Bv2 cells in DMEM that were incubated with or without 200 ng/ml lipopolysaccharide (LPS; Sigma) and 1 unit/ml interferon-γ (IFNγ; R&D Systems, Minneapolis, MN) for 18 h. These conditions were determined in preliminary experiments to cause peak iNOS expression and NO production (Schreiber and El-Fakahany, unpublished observation). The inserts containing the monolayer of RBL-2H3 cells were co-incubated with the Bv2 cells at 37 °C in a shaking water bath (65 rpm) for 30 min. The NO synthase (NOS) inhibitor, L-nitroarginine, was added directly to some of the Bv2-containing wells 15 min prior to co-incubation with RBL-2H3 cells. After the incubation period, the Transwell inserts were removed, washed three times with HEPES buffer, the cells were lysed, and an aliquot of the cell lysate was analyzed for 5-HT content.

2.4. Measurement of L-[3H]citrulline formation

The activity of NOS in Bv2 cells was measured by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. Bv2 cells, grown for 2 days in 24-well plates (to ~40% confluence), were stimulated with cytokines as described above. Following this incubation time, the cells were washed three times with HEPES buffer and L-[2,3,4,5-^{3}H]Arginine monohydrochloride (0.6 μCi/well; 61 Ci/mmol; Amersham, Arlington Height, IL) was added to each well. After a 30-min incubation at 37 °C in a shaking water bath (65 rpm), the reaction was stopped, cells were lysed and L-[^{3}H]citrulline was separated from L-[^{3}H]arginine by anion exchange chromatography as previously described (Wotta et al., 1998).

2.5. Data analysis

Within individual experiments, treatments were repeated in triplicate and results were averaged to obtain one value. Reported data are the mean ± S.E.M. for *n* independent experiments. Statistical analyses were done with one- or two-way ANOVA followed by Student–Newman–Keuls multiple comparisons test, or with Student's unpaired *t*-test. Significance was determined at a level of $P < 0.05$. Nonlinear regression to sigmoidal concentration–response curves and determination of EC_{50} values were performed using Prism (v. 3.0; GraphPad Software, San Diego, CA).

3. Results

3.1. The nitric oxide donor, SNAP, decreases cellular 5-HT content

RBL-2H3 cells synthesize 5-HT, which is stored within granules for release upon stimulation (Horowitz et al., 1982).

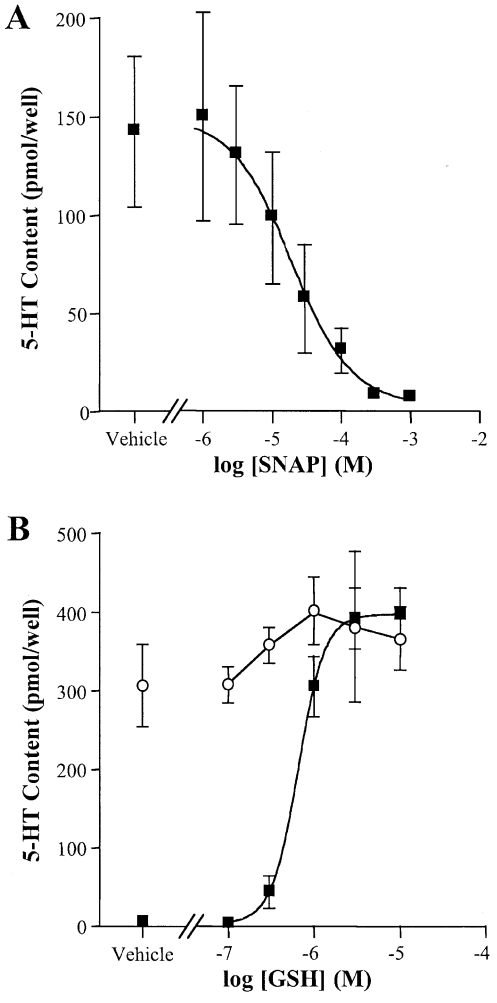

Fig. 1. (A) The nitric oxide donor, SNAP, concentration-dependent depletes the cellular content of 5-HT in RBL-2H3 cells. The experimentally determined EC_{50} value was 18 ± 5 μM. 5-HT levels at SNAP concentrations greater than 10 μM were significantly less than vehicle control values (one-way ANOVA, Student–Newman–Keuls Multiple Comparisons Test). (B) GSH concentration-dependent inhibited the effects of 1 mM SNAP (squares) but did not affect vehicle treated RBL-2H3 cells (circles). Concentrations of GSH greater than 100 nM caused significant reversal of SNAP effects (two-way ANOVA, Student–Newman–Keuls Multiple Comparisons test).

The NO donor, SNAP, was used to assess the action of NO on 5-HT content in these cells. Exposure of RBL-2H3 cells to SNAP caused a concentration-dependent decrease in the content of 5-HT, with an experimentally determined EC_{50} value of 18 ± 5 μM ($n = 3$; Fig. 1A). At a concentration of 1 mM, SNAP decreased 5-HT content to approximately 5% of the control levels.

To determine the Ca^{2+}-dependence of the SNAP-induced decrease in 5-HT content, BAPTA-AM was used under conditions where it chelates intracellular Ca^{2+} (Linden et al., 2000). SNAP (1 mM) significantly reduced 5-HT content to 0.7 ± 0.3 pmol/well, compared to control 5-HT levels of 182 ± 31 pmol/well. BAPTA-AM (100 μM, added for 15 min) did not alter the level of 5-HT in the presence of SNAP (0.9 ± 0.5 pmol/well).

The reduced form of glutathione (GSH), an antioxidant that protects thiol groups from NO through the formation of *S*-nitrosoglutathione (Gow et al., 1997), was tested for its effect on SNAP-induced decrease in 5-HT content. Although no concentration of GSH tested alone altered the content of 5-HT, GSH (5 min prior to SNAP) concentration-dependently suppressed the SNAP-induced decrease in 5-HT content with an experimentally determined IC_{50} value of 670 ± 102 nM ($n = 3$; Fig. 1B).

3.2. Endogenously derived NO decreases cellular content of 5-HT

Bv2 cells, that can be induced to express inducible NOS (iNOS) via stimulation with pro-inflammatory cytokines, were used as an endogenous source of NO. To ensure that LPS and IFNγ treatment for 18 h was an effective stimulus for NO production, the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in these cultures was assayed. Stimulation with the cytokines produced nearly a five-fold increase in NOS activity (Fig. 2A). The NOS inhibitor, L-nitroarginine (100 μM), significantly reduced the production of NO in stimulated Bv2 cells (Fig. 2A). When co-incubated with RBL-2H3 cells, stimulated Bv2 cells caused a significant reduction in the 5-HT content of RBL-2H3 cells when compared to unstimulated Bv2 cells (Fig. 2B). The decrease in 5-HT content in response to endogenously derived NO was similar to RBL-2H3 cells on Transwell inserts that were treated with 1 mM SNAP (91 ± 29 pmol/well). Cytokines in the absence of Bv2 cells had no effect on the 5-HT content of RBL-2H3 cells (161 ± 16 pmol/well; $n = 3$) when compared to control levels (167 ± 27 pmol/well; $n = 3$; $p > 0.05$ *t*-test). L-Nitroarginine attenuated the decrease in 5-HT content to levels that were not different from control values (Fig. 2B).

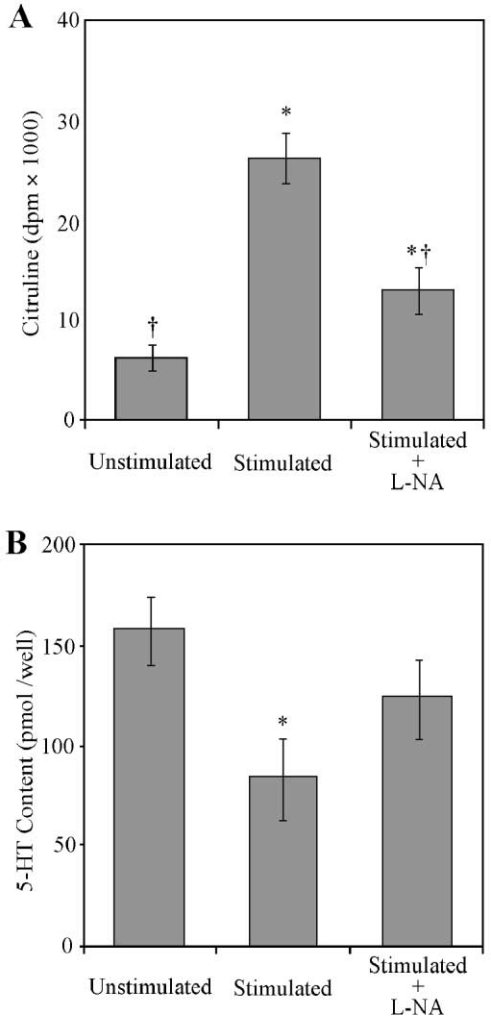

Fig. 2. (A) Bv2 cells stimulated with 200 ng/ml LPS and 1 unit/ml IFNγ generate NO as measured by the accumulation of L-[^{3}H]citrulline. The increase in NO production could be reduced by treatment with the nonselective NOS inhibitor, L-nitroarginine (100 μM). (B) A diffusible messenger from stimulated Bv2 cells causes a reduction of RBL-2H3 cell 5-HT content. L-Nitroarginine attenuates the cytokine-induced reduction in 5-HT content. * Significantly different than unstimulated control values. † Significantly different from stimulated values (one-way ANOVA, Student–Newman–Keuls Multiple Comparisons test).

4. Discussion

The present study provides evidence that NO can deplete naturally stored 5-HT in RBL-2H3 cells. This observation extends a previous report that endogenous NO reduces 5-HT content (Wegener et al., 2000) by providing direct evidence for intercellular interaction between NO and 5-HT. Further study of this model should reveal the mechanism by which NO depletes 5-HT. Several possible mechanisms exist. Because exocytosis of 5-HT from RBL-2H3 cells is a Ca^{2+} dependent process (Kim et al., 1997), and the NO-induced reduction of 5-HT content is independent of intracellular Ca^{2+} concentration (present results), it is unlikely that release is responsible for 5-HT depletion in the current study. A more probable mechanism is an interaction between NO and enzymes responsible for the biosynthesis or catabolism of 5-HT or between NO and 5-HT itself.

NO and NO donors inhibit tryptophan hydroxylase (EC 1.14.16.4), the rate-limiting step in the synthesis of 5-HT (Kuhn and Arthur, 1996, 1997). SNAP was over 200-fold more potent in the current study than the inhibition of isolated tryptophan hydroxylase, but was similarly protected by GSH (Kuhn and Arthur, 1996). The abolition of the SNAP-induced depletion of 5-HT by GSH supports the concept that the NO moiety of SNAP is responsible for the observed effect. However, the action of GSH does not make tryptophan hydroxylase the only viable target of NO. GSH would also protect 5-HT from a direct reaction with NO that forms 4-nitroso-5-HT or 4-nitro-5-HT (Blanchard et al., 1997; Fossier et al., 1999). SNAP-induced depletion of 5-HT here is only 10-fold more potent than NO-induced production of 4-nitroso-5-HT (Fossier et al., 1999), so this mechanism is a likely candidate. Such chemical modifications of 5-HT would not be recognized by the enzyme immunoassay used in the current study and thus would be responsible for the reduction of 5-HT content. Delineation of the mechanism responsible for the observed effect, and the possible involvement of breakdown products of NO requires further exploration.

Modification of 5-HT content in serotonergic pathways by NO can profoundly modulate varied and diverse neural functions. Collectively, the data presented here indicate that endogenously derived NO can reduce the cellular content of 5-HT. Furthermore, the current study describes a novel model system that can be utilized to further study the interaction between the biochemical pathways of the biosynthesis and catabolism of 5-HT and NO. Likewise, this model can be used to develop novel compounds that can modulate the interaction between these transmitter systems. Such compounds may benefit the treatment of disorders where 5-HT transmission is altered such as irritable bowel syndrome, depression and chronic pain.

Acknowledgements

The authors would like to thank Ms. Kristin Schreiber for her valuable input to the development of these studies. This work was supported by the NIH grant NS25743.

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