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SEROTONIN 5HT$_{2A}$ RECEPTOR ACTIVATION INHIBITS INDUCIBLE NITRIC OXIDE SYNTHASE ACTIVITY IN C6 GLIOMA CELLS

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Summary

C6-glioma cells endogenously express both 5HT$_{2A}$ receptors and inducible nitric oxide synthase (iNOS). iNOS can be induced by transcriptional activation to produce nitric oxide (NO) in response to a challenge with lipopolysaccharide (LPS). Experiments were conducted to determine whether 5HT$_{2A}$ receptor activation could modify the production of NO in response to LPS. Incubation of 10μg/ml LPS with C6-glioma cells for a period of 24 hours resulted in a 2.6 fold increase in nitrite levels, as a measure of NO levels, over vehicle treated controls. Co-incubation with the selective 5HT$_{2A}$ receptor partial agonist (±)-2,5-dimethoxy-4-iodoamphetamine (DOI) produced a dose-dependent inhibition of the LPS-induced nitrite levels of 22% with an IC$_{50}$ of 16nM. The full agonists serotonin (5HT) and α-methyl-5HT produced an inhibition of approximately 30% at a concentration of 1μM. The inhibitory effect of 1μM DOI was blocked by the 5HT$_{2A}$ receptor antagonists spiperone and ritanserin (10nM). Inhibition of protein kinase C (PKC) using 100nM chelerythrine prevented the DOI-mediated decrease in LPS-induced nitrite levels. Addition of DOI to the cells after 1hr following the LPS addition did not produce a decrease in nitrite levels indicating iNOS was not modified post-translationally. The data demonstrate that iNOS activity can be modulated by serotonin 5HT$_{2A}$ receptor activation, most likely at the initiation of the induction process, via PKC. We therefore suggest that there may be a link between the serotonergic system and NO-mediated immune responses in the brain.

5-hydroxytryptamine (5-HT, serotonin) is a biogenic amine that functions as a neurotransmitter and hormone. To date at least 14 distinct serotonin receptor subtype cDNAs have been identified which have been classified into seven different sub-families (5HT$_1$-5HT$_7$). The 5HT$_2$ sub-family is composed of the 5HT$_{2A}$, 5HT$_{2B}$ and 5HT$_{2C}$ receptors (1). The 5HT$_2$ receptors are coupled to the

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activation of phospholipase C which initiates the stimulation of
the inositol phosphate and diacylglycerol (IP/DAG) pathway (2-4).
The $5HT_{2A}$ receptors have been implicated in mediating the effects
of indolealkylamine and phenylisopropylamine hallucinogens such as
dimethyltryptamine (DMT) and 2,5-dimethoxy-4-bromoamphetamine
(DOB) respectively, and ergot alkaloids such as LSD (5-7). In
addition this receptor is involved in various behaviors such as a
head twitch response and is believed to participate in the action
of atypical antipsychotics (8, 9).

While the initial signaling events of the individual $5HT_2$
receptors have been well described, the downstream cellular
systems that mediate $5HT_2$ receptor stimulation, following
activation of IP/DAG, have yet to be elucidated. One potential
downstream second messenger system that may be modulated by 5-HT,
and already known to be regulated by protein kinases, is that of
nitric oxide.

Recently nitric oxide (NO) has emerged as a novel second messenger
and neurotransmitter in the CNS (within neurons and glia),
macrophages, endothelial cells and other tissues (10). NO has been
demonstrated to have a number of roles in the brain including
mediation of long-term potentiation (11), regulation of
neurotransmitter transporters (12, 13) as well as neuroprotection
and neurotoxicity (14).

The production of NO is catalyzed by a family of nitric oxide
synthase (NOS) enzymes. Several forms of NOS have been identified
including two constitutive Ca^{++}/calmodulin dependent isoforms,
neuronal NOS and endothelial NOS, as well as a Ca^{++}-independent
isoform termed inducible NOS (iNOS, type II NOS). The iNOS enzyme
is activated at the level of transcription by immunostimulating
compounds such as lipopolysaccharide (LPS) and cytokines such as
IL-1ß and TNF-α (15, 16). Once translated, the iNOS enzyme
produces nitric oxide for a period of hours where it participates
in inflammatory and immune responses (16).

Serotonin has previously been postulated to have immunosuppressant
activity, however the mechanisms of the suppression have not been
fully described at the molecular and cellular level (17-19).
Therefore the purpose of this study was to determine if $5HT_2$
receptor activation can modulate iNOS activity and therefore an
immune response in a glial cell type. We have utilized the C6-
glioma cell line to examine our hypothesis taking advantage of the
fact that this cell line endogenously expresses only the $5HT_{2A}$
subtype of $5HT_2$ receptor family (20) as well as iNOS (16).

Materials and methods

Dulbecco's modified Eagle medium (DMEM), phenol red free DMEM,
phosphate buffered saline, trypsin-EDTA, penicillin/streptomycin,
L-glutamine, serotonin HCl, sodium nitrite, sulfanilamide, N-
napthylethylenediamine and lipopolysaccharide (S. typhimurium)
were all purchased from Sigma (St. Louis, MO). (±)-2,5-dimethoxy-
4-iodoamphetamine (DOI), α-methyl-serotonin, methylergonovine, 5-

carboxyamidotryptamine (5-CT), m-chloro-phenylbiguanide (mCPBG), chelerythrine, spiperone and ritanserin were all purchased from Research Biochemicals Inc. (Natick, MA).

C6-Glioma cells were cultured in $100mm^2$ plates containing 10ml of DMEM media containing 10% fetal bovine serum, 1mM L-glutamine, 100 units penicillin and 100µg/ml streptomycin, were incubated at 37°C and 5% CO_2. Cells were passaged at confluence. Adhered cells were removed with trypsin and plated into 24 well plates at a density of 10^5 cells/well. Twenty-four hours later the cells were switched over to phenol-red free DMEM. The wells were then treated with various drugs at indicated times, with all the media/well harvested for assay at 24 hours following initiation of the 10µg/ml LPS treatment.

NO production was determined by measuring the metabolic products of NO, nitrate and nitrite using the Greiss reagent. Nitrate was first converted to nitrite by incubating the samples with 0.14 units of nitrate reductase in the presence of 10mM NADPH at room temperature for one hour. Equal amounts of Greiss reagent (1.0% sulfanilamide in 5% phosphoric acid and 1.0% n-napthylethylenediamine) were then added, and the absorbance of the azo-dye product determined with a UV/visible spectrophotometer at 540nm (21).

Nitrite standards were prepared from sodium nitrite, ranging in concentration from 0.025µg/ml to 0.75µg/ml. Nitrite concentrations of the samples were determined by linear regression analysis. Protein levels were determined with bicinchoninic acid protein assay reagent using bovine serum albumin as the standard (Pierce). Protein levels were then determined by linear regression analysis.

The IC_{50} values for the above treatments were with Sigma Plot using a Hill plot with a minimum response not equal to zero
$$Y=((A-D)/(1+(X/B)**N))+D$$
where A = maximum response, B = IC_{50}, D= minimum response and N = slope. All results are presented as Means ± SEM of at least three experiments done in triplicate. Statistical differences between two means were assessed by Student's t-test (p<0.05).

Results

Incubation of C6-glioma cells with 10µg/ml LPS resulted in a 2.6 fold increase in nitrite levels (as a measure of iNOS function) as compared to vehicle treated controls (Table 1). The increase in nitrite levels was maximal at 22 hrs, thus for all subsequent experiments we harvested the culture media for assay at 24 hrs post-addition of LPS.

In order to determine if $5HT_{2A}$ receptor activation could modulate the LPS-mediated increase in nitrite levels, we co-incubated various known $5HT_2$ receptor agonists concurrently with the LPS for the 24 hr treatment period. 1µM concentrations of the agonists DOI, 5-HT, α-methyl-5HT and methylergonovine inhibited the LPS-stimulated increase in nitrite (Table 2). The addition of the selective $5HT_3$ receptor agonist m-chloro-phenylbiguanide had no effect on nitrite levels nor did the $5HT_1$ receptor agonist 5-CT.

If the effects of DOI and 5-HT are indeed $5HT_{2A}$ receptor-mediated,

TABLE 1

Incubation Time (Hours)	Nitrite Levels (μg/ml/mg protein)
5 (Vehicle)	0.37±0.07
5 (LPS treated)	0.47±0.03
22 (Vehicle)	0.44±0.05
22 (LPS Treated)	1.17±0.27*

Effect of 5 or 22 hr incubation with 10μg/ml LPS on Nitrite levels in C6 glioma cells. * indicates significantly different from 22 hr vehicle control control (p<0.05, Student's t-test)

TABLE 2

Drug Treatment + 10μg/ml LPS	Nitrite Levels (% of LPS-stimulated Control)
1μM DOI	80±4*
1μM 5-HT	74±6*
1μM α-methyl-5HT	68±6*
1μM Methylergonovine	80±8*
1μM m-Chlorophenylbiguanide	101±7
1μM 5-CT	97±6

Effect of various 5-HT agonists on LPS-stimulated nitrite levels. Results are expressed as the mean±SEM of at least three experiments done in triplicate. * Indicates significantly different from LPS-stimulated control (p<0.05, Student's t-test).

the observed inhibition should be dose-dependent and prevented by low concentrations of high affinity $5HT_2$ receptor antagonists. Dose-response analysis demonstrated that the inhibition produced by both DOI and 5-HT was indeed concentration dependent (Fig. 1).

The IC_{50} values for DOI and 5-HT were 16±3nM and 12±7nM respectively, which resembles their EC_{50} values for the stimulation of IP_3 production in the C6-glioma cell line. Additionally, the inhibition of nitrite levels produced by 1μM DOI was blocked by 10nM spiperone and ritanserin, which are selective $5HT_2$ receptor antagonists, indicating that the effect of DOI was receptor mediated (Table 3). Neither spiperone or ritanserin, at a concentration of 10nM, had an effect on the level of LPS-stimulated nitrite production.

$5HT_{2A}$ receptor activation is known to result in the activation of PKC and inositol phosphate metabolism. As iNOS function is Ca^{++} independent we chose to examine the effect of a PKC inhibitor on the DOI-mediated effect. Addition of 100nM chelerythrine, a selective PKC inhibitor, along with 1μM DOI and 10μg/ml LPS prevented the inhibition produced by DOI (Table 3). Chelerythrine itself did not alter nitrite levels in the presence of LPS. We did not find that phorbol 12-myristate 13-acetate (PMA) had any effect on the LPS-induction of nitrite or on basal nitrite levels in our C6-glioma cell line (data not shown).

In order to determine if the regulation of iNOS is occurring at the level of transcription or post-translationally, we added 1μM
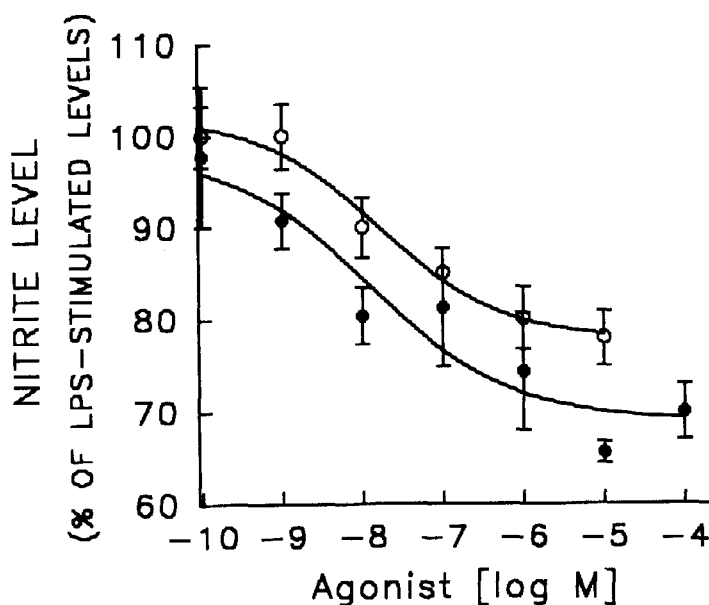
Fig. 1
Dose response analysis of DOI and 5-HT on LPS-stimulated nitrite levels. C6 glioma cells were incubated with the indicated concentrations of DOI (open symbol) or 5-HT (closed symbol) for 24 hrs in the presence of 10µg/ml LPS.

to the cells at various time points before and after the addition of LPS. Additions of DOI at 2 hrs following the addition of LPS did not result in a decrease in nitrite production (data not shown), suggesting the DOI mediated inhibition is occurring at the onset of induction rather than a direct modification of the iNOS enzyme.

The 5HT$_2$ receptor antagonists have been shown to possess inverse agonist activity in some cell types. Therefore we tested higher doses of spiperone for inverse agonist activity. At a concentration of 10µM spiperone produced a potentiation of the LPS-induced increase in nitrite levels (Table 3). The response was not blocked by DOI however. Dose response analysis revealed that the potentiation produced by spiperone, as well as by the antagonists ritanserin and mesulergine, was not dose dependent (data not shown) as only the 10µM dose produced a potentiation. Taken together these results suggest that the potentiation is not receptor mediated.

TABLE 3

Drug Treatment	Nitrite Level (% of LPS-Stimulated Control)
LPS + 1μM DOI	80±4*
LPS + DOI + 10nM Spiperone	95±3
LPS + DOI + 10nM Ritanserin	103±5
LPS + DOI + 100nM Chelerythrine	102±7
LPS + Chelerythrine	96±1
LPS + 10μM Spiperone	181±43*
LPS + 10μM Ritanserin	156±28*
LPS +10μM Mesulergine	181±18*

10μg/ml LPS was incubated with the indicated drug treatments for 24 hrs. * indicates significantly different from LPS-stimulated control ($p < 0.05$, Student's t-test).

Discussion

The results of this study demonstrate that activation of serotonin $5HT_{2A}$ receptors results in the inhibition of iNOS activity in C6-glioma cells. The 2.6 fold increase in nitrite levels produced by 10μg/ml LPS is similar to the response observed by Simmons and Murphy, which assayed cGMP content as a measure of iNOS activity (22). Lower levels of LPS (1μg/ml) have been shown to be ineffective in C6 glioma cells (23). LPS injected i.p. has previously been shown to induce iNOS activity in the brain, therefore LPS activation of glia is physiologically possible (24). We are currently examining the regulation of 5HT2A receptor stimulation on cytokine mediated iNOS induction, which gives a >10 fold increase in nitrite levels.

The maximum decreases produced by 5-HT and α-methyl-5HT coincide with the full agonist profile of these compounds at the $5HT_{2A}$ receptor (25). Similarly the partial agonist effect of DOI is consistent with its known efficacy in other models of $5HT_2$ receptor activity (26). The dose dependency, IC_{50} values and antagonist inhibition of the DOI-mediated effect further demonstrates that the observed response is $5HT_{2A}$ receptor mediated. It is important to note that the serum used in our culture media is likely to have contained serotonin, which could skew our IC_{50} estimates. When we attempted these experiments in serum free media, however, we failed to obtain the LPS-induction of nitrite. Perhaps a lower level of serum or charcoal stripping the serum could eliminate this problem.

The inhibitory effect of chelerythrine on the DOI-induced inhibition of iNOS function demonstrates that PKC is mediating the effect of $5HT_{2A}$ receptor activation. PMA has been shown to modulate iNOS activity on other cell lines being dependent on the subtypes of PKC present in the cells and the time course of treatment (27). Indeed, Syapin (28) co-administered PMA with LPS and observed a synergistic effect on the increase in nitrite levels. As stated above, however, we did not observe this effect in our C6 glioma cell line.

The time course analysis suggests that the inhibition of iNOS

function is occurring at the level of enzyme induction rather than as a direct modification of the iNOS protein. The initiation of iNOS induction occurs immediately following LPS addition. iNOS activity is then detectable 2-3 hrs after the addition of LPS (29). The failure of DOI to inhibit the increase in nitrite levels at two hours following the addition of LPS indicates that regulation is occurring during the induction process. If a post-translational modification occurred, addition of DOI at any time after 1-2 hr would have resulted in an observed decrease in nitrite levels. It is not possible to determine the exact point at which the induction process is inhibited by DOI. Western Blot analysis will be useful in further clarifying these observations as a decrease in the level of induction should result in a decrease in detectable iNOS protein levels.

LPS is believed to initiate the induction process via activation of a tyrosine kinase (23). Subsequently nuclear factor kappa-B (NF-κB) is activated leading to the onset of transcription. It is possible that one of these proteins is being modified by the $5HT_{2A}$ receptor activation of PKC. Alternatively the translational machinery could be altered by PKC. Analysis of these individual pathway factors will be necessary to determine the exact mechanism of the $5HT_{2A}$ receptor-mediated decrease in iNOS function. Other neurotransmitters/hormone systems have been shown to inhibit iNOS activity and expression by these mechanisms. Cannabinoid receptor activation results in a decrease in iNOS expression and an approximate decrease in nitrite levels of 30%, which has been demonstrated to occur via the inactivation of the NF-kB/Rel family of transcription factors in RAW 264.7 cells (30). The inhibition of iNOS by cannabinoids in these cells was not affected by PMA, similar to our results. Angiotensin II receptor activation resulted in a 70% decrease in nitrite formation and decreased iNOS expression in vascular smooth muscle cells (31). The efficacy of angiotensin II was diminished when added more than three hrs following the initial start of induction, demonstrating that the time course analysis can differentiate transcriptional regulation from post-translational modification.

Evidence has been accumulating that classical neurotransmitters and various cytokine systems can regulate one another (32). Indeed, $5HT_2$ receptor activation has previously been shown to modify immune responses, including inhibition of tumor necrosis factor-α synthesis in human monocytes and there is evidence of $5HT_2$ receptors on murine lymphocytes (17), and LPS activation of IL-1 has been shown to regulate serotonin metabolism as well(33).

The findings presented here demonstrate that serotonin can partially inhibit the induction of iNOS in C6 glioma cells. Therefore we suggest that serotonin may act as an immunosuppressor in the brain and that serotonergic activation of $5HT_{2A}$ receptors could potentially suppress the inflammatory responses produced by LPS, ischemia and stroke.

References

1. D. HOYER, D.E. CLARKE, J.R. FOZARD, P.R. HARTIG, G.R. MARTIN, E.J. MYLECHRANE, P.R. SAXENA and P.P.A. HUMPHREY. Pharmacol. Rev. **46** 157-203 (1994).

2. P.J. CONN and E. SANDERS-BUSH. Neuropharmacology **23** 993-996 (1984).

3. J.D. KURSAR, D.L. NELSON, D.B. WAINSCOTT, M.L. COHEN and M. BAEZ. Mol. Pharmacol. **42** 549-557 (1992).

4. P.J. CONN, E. SANDERS-BUSH, B.J. HOFFMAN and P.R. HARTIG. Proc. Natl. Acad. Sci. (USA) **83** 4086-4088 (1986).

5. M. TEITLER, R.A. LYON and R. A. GLENNON. Psychopharmacology **94** 213-216 (1988).

6. E. SANDERS-BUSH and M. BREEDING. Psychopharmacology **105** 340-346 (1991).

7. R.A. GLENNON, M. TEITLER and J.D. McKENNEY. Life Sci. **35** 2205-2211 (1984).

8. R. SCHREIBER, M. BROCCO, V. AUDINOT, A. GOBERT, S. VEIGA and M.J. MILLAN. J. Pharmacol. Exp. Ther. **273** 101-112 (1995).

9. H.Y. MELTZER, S. MATSUBARA and J.C. LEE. J. Pharmacol. Exp. Ther. **251** 238-246 (1989).

10. T.M. DAWSON AND S.H. SNYDER. J. Neurosci. **14** 5147-5159 (1990).

11. M. ZHOU, S. SMALL, E.R. KANDEL and R.D. HAWKINS. Science **260** 1946-1950 (1993).

12. K.J. MILLER and B.J. HOFFMAN. J. Biol. Chem. **269** 27351-27356 (1994).

13. S. POGUN, M.H. BAUMAN and M.J. KUJAR. Brain Res. **641** 83-91 (1994).

14. S.A. LIPTON, Y.B. CHOI, Z.H. PAN, S.Z. LEI, H.Z. CHEN, N.J. LOSCALZO, D.J. SINGEL and J.S. STAMLER. Nature **364** 626-632 (1993).

15. C.J. LOWENSTEIN, C.S. GLATT, D.S. BREDT and S.H. SNYDER. Proc. Natl. Acad Sci. (USA) **89** 6711-6715 (1992).

16. C.J. LOWENSTEIN, E.W. ALLEY, P. RAVAL, A.M. SNOWMAN, S.H. SNYDER, J. RUSSEL and W.J. MURPHY. Proc. Natl. Acad. Sci. (USA) **90** 9730-9734 (1993).

17. E. ARTZ, M. COSTAS, S. FINKIELMAN and V.E. NAHMOD. Life Sci. **48** 2557-2562 (1991).

18. J.C. AMEISEN, R. MEADE and P.W. ASKENASE. J. Immunol. **142** 3171-3179 (1989).

19. D.O. SLAUSON, C. WALKER, F. KRISTENSEN, Y. WANG and A.L. DE WECK. Cell. Immunol. **84** 240-252 (1984).

20. J. M. ELLIOT, N. R. NEWBERRY, A. J. CHOLEWINSKI, J. T. ARTRUP, S. J. BRIDDON, J. E. CAREY, T. P. FLANIGAN, R. A. EWTON, S. L. PHIPPS, A. C. REAVLEY ET AL. Neuroscience 69 119-1131 (1995).

21. L.C. GREEN, D.A. WAGNER, J. GLOGOWSKI, P.L. SKIPPER, J.S. WISHNOOK and S.R. TANNENBAUM. Analytical Biochem. **126** 131-138 (1982).

22. M. L. SIMMONS and S. MURPHY. J. Neurochem. **59** 897-905 (1992).

23. D.L. FEINSTEIN, E. GALEA, J. CERMAK, P. CHUGH, L. LYANDVERT and D.J. REIS. J. Neurochem. **62** 811-321 (1994).

24. A. J. DUNN. J. Pharmacol. Exp. Ther. **267** 964-969 (1992).

25. E. EDWARDS, C.R. ASHBY and R.Y. WANG. Neuropharmacology **31** 615-621 (1992).

26. J.A. APUD, D.R. GRAYSON, E. DE ERAUSQUIN and E. COSTA. Neuropharmacology **31** 1-8 (1992).

27.   A. PAUL, K. DOHERTY and R. PLEVIN. Br. J. Pharmacol. **120**
      940-946 (1997).
28.   P. J. SYAPIN. Alchohol Clin. Exp. Res. **19** 262-267 (1995).
29.   C. NATHAN and Q. XIE. Cell **78** 915-918 (1994).
30.   Y.J. JEON, K.H. YANG, J.T. PULASKI and N.E. KAMINSKI. Mol.
      Pharmacol. **50** 334-341 (1996).
31.   I. NAKAYAMA, Y. KAWAHARA, T. TSUDA, M. OKUDA and M.
      YOKOYAMA. J. Biol. Chem. **269** 11628-11633 (1994).
32.   F. BERKENBOSCH, D.E.C. DE GOEIJ, A. DEL REY AND H.O.
      BESEDOVSKY. Neuroendocrinology **50** 570-576 (1989).
33.   A.J. DUNN. J. Pharmacol. Exp. Ther. **356** 964-969 (1992).