Hypoxia

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ORIGINAL RESEARCH

Hypoxia attenuates purinergic P2X receptor-induced inflammatory gene expression in brainstem microglia

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Abstract: Hypoxia and increased extracellular nucleotides are frequently coincident in the brainstem. Extracellular nucleotides are potent modulators of microglial inflammatory gene expression via P2X purinergic receptor activation. Although hypoxia is also known to modulate inflammatory gene expression, little is known about how hypoxia or P2X receptor activation alone affects inflammatory molecule production in brainstem microglia, nor how hypoxia and P2X receptor signaling interact when they occur together. In the study reported here, we investigated the ability of a brief episode of hypoxia (2 hours) in the presence and absence of the nonselective P2X receptor agonist 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP) to promote inflammatory gene expression in brainstem microglia in adult rats. We evaluated *inducible nitric oxide synthase* (*iNOS*), *tumor necrosis factor alpha* (*TNFα*), and *interleukin (IL)-6* messenger RNA levels in immunomagnetically isolated brainstem microglia. While *iNOS* and *IL-6* gene expression increased with hypoxia and BzATP alone, *TNFα* expression was unaffected. Surprisingly, BzATP-induced inflammatory effects were lost after hypoxia, suggesting that hypoxia impairs proinflammatory P2X-receptor signaling. We also evaluated the expression of key P2X receptors activated by BzATP, namely *P2X1*, *P2X4*, and *P2X7*. While hypoxia did not alter their expression, BzATP upregulated *P2X4* and *P2X7* mRNAs; these effects were ablated in hypoxia. Although both *P2X4* and *P2X7* receptor expression correlated with increased microglial *iNOS* and *IL-6* levels in microglia from normoxic rats, in hypoxia, *P2X7* only correlated with *IL-6*, and *P2X4* correlated only with *iNOS*. In addition, correlations between *P2X7* and *P2X4* were lost following hypoxia, suggesting that *P2X4* and *P2X7* receptor signaling differs in normoxia and hypoxia. Together, these data suggest that hypoxia suppresses P2X receptor-induced inflammatory gene expression, indicating a potentially immunosuppressive role of extracellular nucleotides in brainstem microglia following exposure to hypoxia.

Keywords: P2X4, P2X7, P2X1, BzATP, inflammation, cytokine

Introduction

Microglia are immune cells resident in the central nervous system (CNS) that continuously survey their environment and respond to changes in cellular homeostasis resulting from infection, hypoxia, cell death, and other stimuli to produce inflammatory molecules that are ultimately thought to be detrimental to neurons. Although the mechanisms activating microglia have been widely studied, little is known concerning the role of extracellular nucleotides, including adenosine diphosphate and adenosine triphosphate (ATP), in microglial activation and transcription of inflammatory genes in vivo. Extracellular nucleotides and their interactions with P2X and P2Y purinergic receptors are important signals permitting microglia to sense and respond to their local CNS environment.[1,2] Nucleotides are co-packaged with neurotransmitters[3] and released

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from astrocytes during calcium wave propagation,[4,5] perhaps enabling microglia to sense synaptic health.[6,7] Nucleotides also leak from damaged and/or dying cells,[3] creating extracellular ATP concentrations sufficient to induce inflammatory activities via P2X7 receptor activation.[8]

Many disorders accompanied by microglial inflammation and high extracellular adenine nucleotide levels (ie, cell death) are associated with hypoxia. For example, hypoxia is an element of ischemic injuries during stroke or myocardial infarction. Another example is the chronic intermittent hypoxia (repeated hypoxia/re-oxygenation events) experienced during sleep-disordered breathing, a frequent occurrence in many neurodegenerative, traumatic, and genetic CNS disorders.[9–14] Although nucleotides and hypoxia each regulate microglial inflammatory activities (reviewed in Di Virgilio et al[15] and Deng et al),[16] little is known concerning their interactions in regulating microglial activities when hypoxia and increased extracellular nucleotides occur together. These microglial stimuli are often coincident in pathology[17–21] and during normal CNS function in hypoxia-sensitive CNS regions, such as the brainstem where hypoxia-induced ATP release is important for maintaining respiration.[22] Thus, we investigated the effects of a brief 2-hour period of hypoxia in the presence and absence of P2X receptor activation on microglial inflammatory gene expression in vivo. Specifically, we tested the hypotheses that P2X receptor activation stimulates microglial inflammatory gene expression in normoxia, and that these effects would be potentiated in hypoxia.

In microglia, many immunomodulatory effects of ATP are mediated through the P2X receptor family member P2X7.[23–26] While 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP) is often regarded as a specific P2X7 receptor agonist, it has at least some potency at all P2X receptor subtypes with the exception of P2X6.[27] Here, we treated rats intracisternally with BzATP and then exposed them to hypoxia or normoxia for 2 hours, followed by returning them to room air. We then evaluated the expression of several inflammatory genes – *inducible nitric oxide synthase* (*iNOS*), *interleukin-6* (*IL-6*) and *tumor necrosis factor alpha* (*TNFα*) – in freshly isolated microglia. We chose iNOS as an endpoint because the inhibition or genetic deletion of this enzyme ameliorates brain damage in multiple ischemic, excitotoxic, and hypoxic injury models.[28–32] The pro-inflammatory cytokines TNFα and IL-6 were chosen because their upregulation is a hallmark of neuroinflammation, and they are often implicated in neuronal toxicity following many CNS insults, including hypoxia/ischemia, neurodegeneration, and traumatic injury (reviewed in Kraft et al,[33] Spooren et al,[34] Smith et al,[35] and Lenzlinger et al).[36]

Materials and methods

Materials

BzATP was purchased from Sigma-Aldrich (St Louis, MO, USA).

Animals

Experiments were performed using 3- to 5-month-old adult male Sprague Dawley rats (Harlan Laboratories, Madison, WI, USA). Animals were maintained in an animal facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International according to protocols approved by the University of Wisconsin Institutional Animal Care and Use Committee. All animals were housed under standard conditions, with a 12-hour light–dark cycle and food and water ad libitum. All efforts were made to minimize animal distress and reduce the number used, while permitting the formation of statistically reliable conclusions.

Methods

Nucleotide (BzATP) treatment

Rats were naive (n = 6) or treated intracisternally with vehicle (25 µL of 250 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]) or BzATP (25 µL of 0.3 mM stock in 250 mM HEPES). Briefly, rats were pre-sedated subcutaneously with dexmedetomidine (50–65 µg/kg), anesthetized with isoflurane (1.5%, 100% O_2 balanced), orotracheally intubated and ventilated (using a Small Animal Ventilator 683, Harvard Apparatus, Inc., Holliston, MA, USA). A tail vein catheter was inserted to deliver fluids (Lactated Ringer's solution, 2.5 mL per hour, intravenously) into the animal post-injection. After dorsal laminectomy at C2, the dura was cut to allow insertion of a silicone catheter into the cisterna magna (12 mm, inserted from the caudal end of the C1 vertebrae) through which the vehicle or BzATP was delivered. The muscle and the skin were sutured closed, and atipamezole (500 µg/kg, intramuscularly), buprenorphine (0.05 mg/kg, subcutaneously), and enrofloxacin (10 mg/kg, subcutaneously) were administered prior to termination of isoflurane anesthesia.

In vivo hypoxia exposure

Exposure was performed by placing animals into individual chambers connected to a computer-driven controller that monitored O_2 and CO_2 within the exposure chamber and

mixed O_2 and/or N_2 to achieve the desired inspired oxygen concentrations, with a CO_2 concentration <0.5%. Animals were exposed to either normoxia (vehicle, n = 8; BzATP, n = 8) or hypoxia (vehicle, n = 7; BzATP, n = 7) for 2 hours, with free access to food and fluids. This paradigm of hypoxia induces a rapid decrease in oxygen tension in the CNS,[37] exerts measurable physiological changes in brainstem neuron excitability,[38–40] and promotes translocation to the nucleus of hypoxia inducible factor-1α.[41] In addition to vehicle-treated rats, naive rats were also exposed to normoxia to control for nonspecific, surgically-induced inflammation. At the end of the 2-hour exposure period, the rats were removed from the chambers, their tail vein catheter was removed, and they were returned to their cages for 22 hours. At that time, the rats were euthanized, and brainstem microglia were immunomagnetically isolated for analysis of gene expression by quantitative real-time polymerase chain reaction (qRT-PCR).

Immunomagnetic CD11b$^+$ cell isolation

CD11b$^+$ cells were isolated from the brainstems of 6–13 individual animals per treatment group, as we have previously reported.[42,43] The average purity of cells isolated from these animals which had the characteristics of microglia was >95% as determined by forward/side scatter analysis and CD11b$^+$/CD45^{low} staining [42,43] (data not shown), consistent with previous reports.[44] These CD11b$^+$ cells are subsequently referred to as "microglia" in this paper.

qRT-PCR

Total RNA was isolated from freshly isolated brain-stem microglia (or whole brain as a positive control for primer-set validation) using Sigma-Aldrich TRI Reagent® according to the manufacturer's instructions. Purified RNA was then digested with DNase I (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and an oligo(dT)/random hexamer cocktail (Promega, Fitchburg, WI, USA). The cDNA was used for qRT-PCR using Power SYBR (Applied Biosystems, Foster City, CA, USA). Fluorescence was monitored in real-time using the TaqMan ABI 7300 Sequence Detection System (Applied Biosystems). The standard curve method[45] was used to determine the relative amounts of each gene between samples using the average of duplicate interpolated C_T values normalized to 18s ribosomal RNA (rRNA). A two-way analysis of variance (ANOVA) was used to determine whether treatment had any effect on the expression of 18s; we found no statistically significant differences in any treatment group (data not shown). The following primer sequences had efficiencies >97%, and were used for qRT-PCR (GenBank® accession numbers are provided in parentheses): iNOS (NM_012611.3), 5′ AGG GAG TGT TGT TCC AGG TG and 5′ TCT GCA GGA TGT CTT GAA CG; IL-6 (NM_012589.2), 5′ GTG GCT AAG GAC CAA GAC CA and 5′ GGT TTG CCG AGT AGA CCT CA; TNFα (NM_012675.3), 5′ TCC ATG GCC CAG ACC CTC ACA C and 5′ TCC GCT TGG TGG TTT GCT ACG; P2X1 (NM_001142367.1), 5′ AGC CCA AGG TAT TCG CAC AG and 5′ TTC ACA GTG CCA TTG AAG GG; P2X2 (NM_053656.2), 5′ GTA GTC AGC ATC ATC ACC AGG and 5′ TCA GAC AAG TCC AGG TCA CAG T; P2X3 (BC081783.1), 5′ TAC CAA GTC GGT GGT TGT GA and 5′ CCA CCC CAC AAA GTA GGA GA; P2X4 (NM_031594.1), 5′ GTG GCG GAC TAT GTG ATT CC and 5′ GGT GCT CTG TGT CTG GTT CA; P2X5 (NM_080780.2), 5′ TCT TGC ATC CAG TGA AGA CG and 5′ AGT TCA GAG CTG TGG CCT GT; P2X6 (NM_012721.2), 5′ ACG TGT TCT TCC TGG TAA CCA ACT and 5′ TGG ACA TCT GCC CTG GAC TT; P2X7 (NM_019256.1), 5′ GGC ACC ATC AAG TGG ATC TT and 5′ CTT GTC GCT CAT CAA AGC AA; and 18s (NR_046237.1), 5′ CGG GTG CTC TTA GCT GAG TGT CCC G and 3′ CTC GGG CCT GCT TTG AAC AC. All primers were designed to span introns whenever possible, and primer efficiency was tested by serial dilutions in standard curves. Primer specificity was assessed using the NCBI (National Center for Biotechnology Information) BLAST® (Basic Local Alignment Search Tool) prior to use, and all dissociation curves had a single peak with an observed T_m consistent with the intended amplicon sequences. Samples with C_T values ≥34 cycles were considered undetectable, and were removed from statistical analyses.

Statistical analyses

Statistical analyses were performed on the normalized, interpolated C_T values from the standard curves from each gene, as previously described.[45] Outliers (identified using Grubb's outlier test) were removed from the dataset. When comparing two population means, statistical inferences were made using a Student's *t*-test. When comparing treatment and oxygen effects, comparisons were made by two-way ANOVA (using Sigma Stat; v 11, Systat Software, San Jose, CA, USA); Tukey post hoc tests were used to assess statistical significance in individual comparisons. Datasets that failed normality were logarithmically transformed prior to running the statistical analyses. Statistical significance was

set at $P < 0.05$. There was no significant difference in gene expression between vehicle-treated and naive normoxic animals for all genes studied, as determined by Student's *t*-test (data not shown). Therefore, these groups were combined for subsequent statistical and graphical purposes. Mean data are expressed ±1 standard error of the mean.

Results

mRNA levels of pro-inflammatory genes are differentially increased by hypoxia in microglia

Hypoxia increased both *iNOS* and *IL-6* mRNA levels (2.57 ± 0.63-fold, $P = 0.02$ and 2.77 ± 0.56-fold, $P = 0.04$, respectively) in freshly isolated brainstem microglia (Figure 1A and B). Interestingly, *TNFα* mRNA levels were not changed by hypoxia (0.89 ± 0.35-fold, $P > 0.05$) (Figure 1C), suggesting that the pro-inflammatory effects of hypoxia are gene specific. Thus, hypoxia increases expression of some but not all pro-inflammatory genes in brainstem microglia in vivo.

P2X receptor activation in normoxia upregulates inflammatory gene expression in brainstem microglia

To investigate the effects of P2X receptor activation on microglial inflammatory gene expression, rats were intracisternally injected with either vehicle or BzATP, and exposed to normoxia or hypoxia. Similar to hypoxia alone, BzATP increased microglial *iNOS* (6.73 ± 2.17-fold, $P < 0.001$) and *IL-6* (2.32 ± 0.53-fold, $P = 0.01$) mRNA levels, but not *TNFα* (1.47 ± 0.24-fold, $P > 0.05$), demonstrating gene-specific regulation of pro-inflammatory molecules by P2X receptors (Figure 1A–C). The stimulatory effects of BzATP on microglial *IL-6* gene expression in vivo are consistent with our previous observations in vitro,[46] where BzATP was found to increase *IL-6* mRNA levels.

BzATP-induced inflammation is prevented by exposure to hypoxia

Surprisingly, the effects of P2X receptor activation on microglial inflammatory gene expression in normoxia were lost in hypoxia. In hypoxia, BzATP failed to increase *iNOS*, *IL-6*, or *TNFα* mRNA levels compared with vehicle treatment (Figure 1). The approximate seven-fold increase in *iNOS* expression stimulated by BzATP in normoxia was reduced by more than half (2.37 ± 0.87-fold, $P = 0.039$) in hypoxia, and was not different from the effects of hypoxia alone (2.57 ± 0.63-fold, $P = 0.536$). Similarly, the ~two-fold increase in *IL-6* mRNA levels stimulated by BzATP in normoxia appeared to decrease (to 0.92 ± 0.25-fold), although these apparent changes were not significant in two-way ANOVA ($P = 0.193$). However, there was a significant

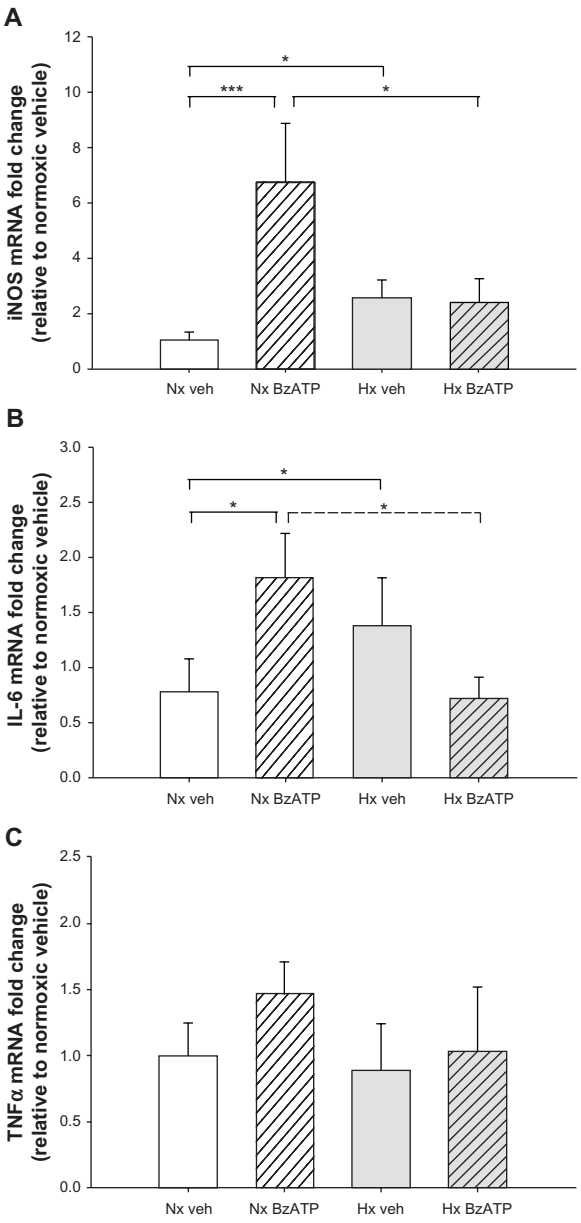

Figure 1 Effects of 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP) treatment and hypoxia exposure on microglial inflammatory gene expression. Brainstem microglia were immunomagnetically isolated from animals treated with vehicle (Veh) or BzATP and exposed to normoxia (Nx) or hypoxia (Hx). Total RNA was isolated and subjected to quantitative real-time polymerase chain reaction for analysis of: (**A**) inducible nitric oxide synthase (iNOS), (**B**) interleukin (IL)-6, and (**C**) tumor necrosis factor alpha (TNFα). Hypoxia for 2 hours increased the expression of iNOS and IL-6. BzATP treatment also increased iNOS and IL-6 expression in normoxia, but these effects were prevented in hypoxia. Neither treatment affected TNFα gene expression.
Notes: Solid lines indicate statistically significant differences with a two-way analysis of variance and dashed lines with a *t*-test. *$P < 0.05$; ***$P < 0.001$. Data are graphed as fold change relative to vehicle treatment.
Abbreviations: mRNA, messenger RNA; RNA, ribonucleic acid.

difference between BzATP effects in normoxia and hypoxia (Student's *t*-test; $P = 0.045$). BzATP effects on *IL-6* expression during hypoxia were not different from vehicle ($P = 0.409$). Further, BzATP had no effect on *TNFα* mRNA expression ($P > 0.05$) with hypoxia. Collectively, these data suggest that P2X receptor function differs in normoxia and hypoxia, and that the ability of P2X receptor signaling to induce inflammatory gene expression in microglia is ablated by hypoxia.

BzATP increases P2X4 and P2X7 mRNA levels

We evaluated the expression of all mammalian P2X receptors in brainstem microglia to narrow the potential list of P2X receptors that could be mediating the BzATP effects. The average C_T values for P2X receptors in brainstem microglia are as follows: P2X1, 29.89; P2X2, ND; P2X3, 30.34; P2X4, 28.14; P2X5, 30.48; P2X6, ND; P2X7, 28.15; and 18s rRNA, 14.41. As P2X7, P2X4, and P2X1 receptors are the most highly expressed P2X receptors in brainstem microglia, and BzATP has the highest affinity for these same receptors,[27] we evaluated BzATP effects on the expression of these receptors in normoxia versus hypoxia (Figure 2). Hypoxia alone had no effect on *P2X7* (1.51 ± 0.38-fold, $P = 0.217$) (Figure 2A), *P2X4* (0.48 ± 0.13-fold, $P = 0.565$) (Figure 2B), or *P2X1* (0.41 ± 0.18-fold, $P > 0.05$) (Figure 2C) expression. In normoxia, BzATP increased expression of both *P2X4* (4.12 ± 1.42-fold, $P = 0.002$) and *P2X7* (3.03 ± 0.70-fold, $P = 0.002$) receptors, but not of *P2X1* receptors (0.78 ± 0.70-fold, $P > 0.05$). As observed with the inflammatory genes, the effects of BzATP on *P2X4* (1.05 ± 0.31-fold, $P < 0.001$) and *P2X7* (0.23 ± 0.07-fold, $P < 0.001$) mRNA levels were lost in hypoxia (Figure 2A and B). Interestingly, while the effects of BzATP in hypoxia on *P2X4* and *P2X7* mRNA levels were not different from the vehicle ($P = 0.227$ and $P = 0.509$, respectively), BzATP increased *P2X1* mRNA levels ($P = 0.024$) in hypoxia (Figure 2C).

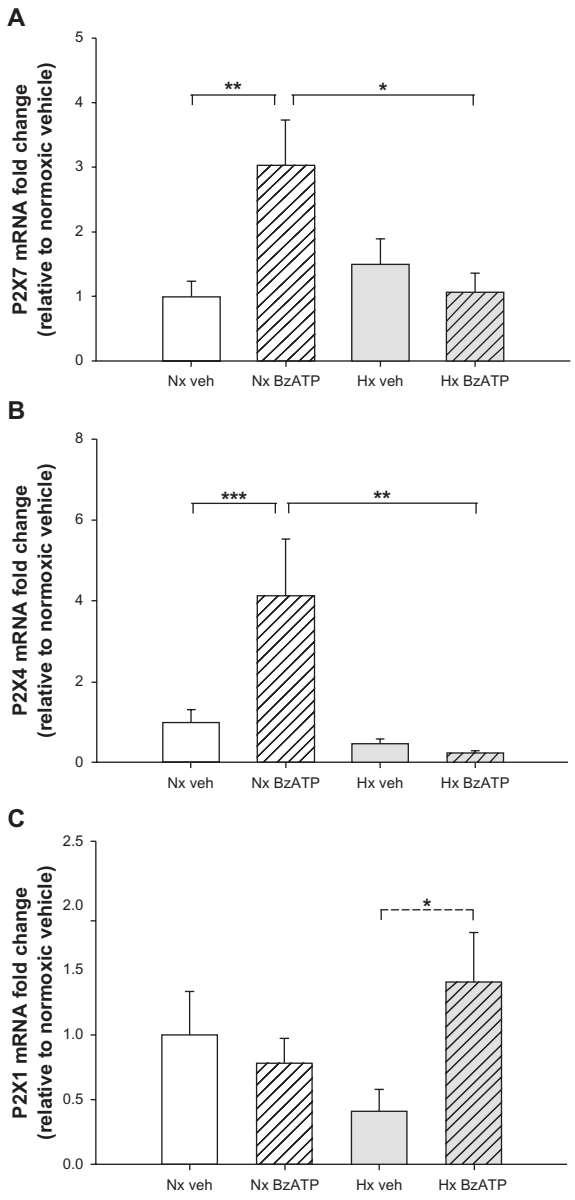

Figure 2 Effects of 2′(3′)-O-(4-benzoylbenzoyl)adenosine-5′-triphosphate (BzATP) treatment and hypoxia exposure on microglial P2X receptor gene expression. Brainstem microglia were immunomagnetically isolated from animals treated with vehicle (Veh) or BzATP and exposed to normoxia (Nx) or hypoxia (Hx). Total RNA was isolated and subjected to quantitative real-time polymerase chain reaction for analysis of: (**A**) P2X7, (**B**) P2X4, and (**C**) P2X1 receptors. Whereas hypoxia alone had no effect on P2X receptor expression, BzATP treatment increased P2X7 and P2X4 expression in normoxia, effects that were prevented in hypoxia. Neither BzATP treatment in normoxia, nor hypoxia exposure affected P2X1 gene expression, but BzATP increased P2X1 mRNA levels in hypoxia.
Notes: Solid lines indicate statistically significant differences determined by two-way analysis of variance and dashed lines by a *t*-test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Data are graphed as fold change relative to vehicle treatment.
Abbreviations: mRNA, messenger RNA; RNA, ribonucleic acid.

P2X receptor expression correlates with inflammatory gene expression in brainstem microglia

Due to the similarities between the regulation of inflammatory genes, *P2X4* and *P2X7* receptors in normoxia, and hypoxia by BzATP, we sought to determine if correlations existed between these genes using regression analyses. We observed strong, positive correlations between both *P2X7* and *P2X4* receptors and *iNOS* (Figure 3A and B) and *IL-6* (Figure 3C and D) expression, although some correlations differed in normoxia and hypoxia. In normoxia, *iNOS* mRNA correlated with both *P2X7* (Figure 3A; $R^2 = 0.480$, $P = 0.003$) and *P2X4* (Figure 3B) mRNA levels ($R^2 = 0.644$, $P < 0.001$), although the correlation was stronger with *P2X7* (correlation coefficient [r] = 0.936) than with *P2X4* (r = 0.771). In contrast, the *iNOS* correlation with *P2X7* mRNA was lost in hypoxia

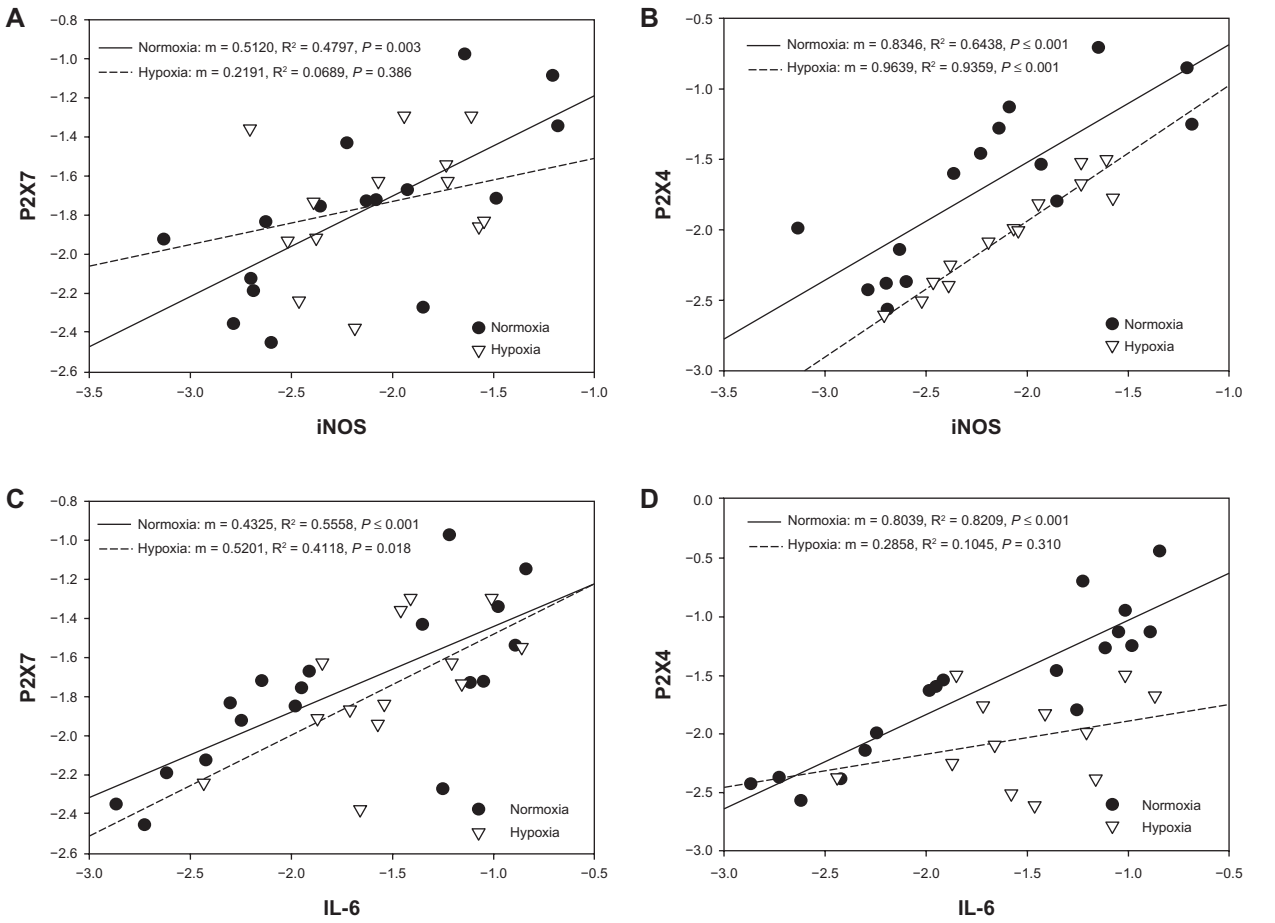

Figure 3 Correlations between P2X4 and P2X7 receptor messenger RNAs (mRNAs) and inducible nitric oxide synthase (iNOS) and interleukin (IL)-6 gene expression differ in normoxia and hypoxia. Multiple linear regression analyses were performed on inflammatory and P2X-receptor gene expression data (logarithmically transformed interpolated C_T values) in normoxia and hypoxia. Correlations between (**A**) iNOS and P2X7, (**B**) iNOS and P2X4, (**C**) IL-6 and P2X7, and (**D**) IL-6 and P2X4 are shown. iNOS expression significantly correlated with both P2X7 and P2X4 receptors in normoxia, whereas, in hypoxia, correlation with P2X7 was lost. Likewise, IL-6 expression correlated with both P2X4 and P2X7 in normoxia, but correlation with P2X4 was lost in hypoxia.
Abbreviation: RNA, ribonucleic acid.

($R^2 = 0.0689$, $P < 0.386$), whereas the correlation with *P2X4* was increased ($R^2 = 0.936$, r = 0.971, $P < 0.001$).

Like *iNOS*, *IL-6* positively correlated with both *P2X7* (Figure 3C; $R^2 = 0.556$, r = 0.936, $P < 0.001$) and *P2X4* expression (Figure 3D) ($R^2 = 0.821$, r = 1.02, $P < 0.001$) in normoxia. However, the correlation with *P2X4* was lost in hypoxia ($R^2 = 0.105$, r = 0.359, $P = 0.310$), whereas the correlation with *P2X7* remained intact ($R^2 = 0.412$, r = 0.314, $P = 0.018$). *IL-6* regulation by *P2X7* in vivo is consistent with our previous in vitro observations using *P2X7* RNA interference[46] where we found that P2X7 receptor knockdown in N9 microglia ablated more than 90% of the BzATP-induced upregulation of *IL-6*. In the present study, no correlation was observed between *TNFα* and either *P2X4* or *P2X7* (data not shown), consistent with the lack of effect of BzATP on *TNFα* gene expression in any oxygen condition. In addition, *P2X1* expression did not correlate with *iNOS*, *TNFα*, or *IL-6* (data not shown). Interestingly, the expression of *P2X4* and *P2X7* receptors was strongly correlated in normoxia (Figure 4A; $R^2 = 0.815$, r = 1.311, $P < 0.001$) but not in hypoxia ($R^2 = 0.139$, r = 0.412, $P = 0.233$), suggesting an "uncoupling" of *P2X4* and *P2X7* regulation by hypoxia.

P2X1 mRNA did not correlate with *P2X4* (Figure 4B; $R^2 = 0.0097$, r = 0.119, $P = 0.773$) or *P2X7* (Figure 4C; $R^2 = 0.020$, r = 0.17, $P = 0.658$) in normoxia. In hypoxia, there was a marginal negative correlation between *P2X1* and *P2X4* expression ($R^2 = 0.346$, r = –0.761, $P = 0.073$, n = 10) and a significant correlation with *P2X7* mRNA ($R^2 = 0.632$, r = –1.283, $P = 0.006$), suggesting that hypoxia may alter P2X receptor signaling by shifting the relative balance of P2X receptors.

Discussion

To our knowledge, this is the first report to describe the effects of P2X receptor activation and hypoxia on microglial inflammatory and P2X receptor gene expression in vivo. We

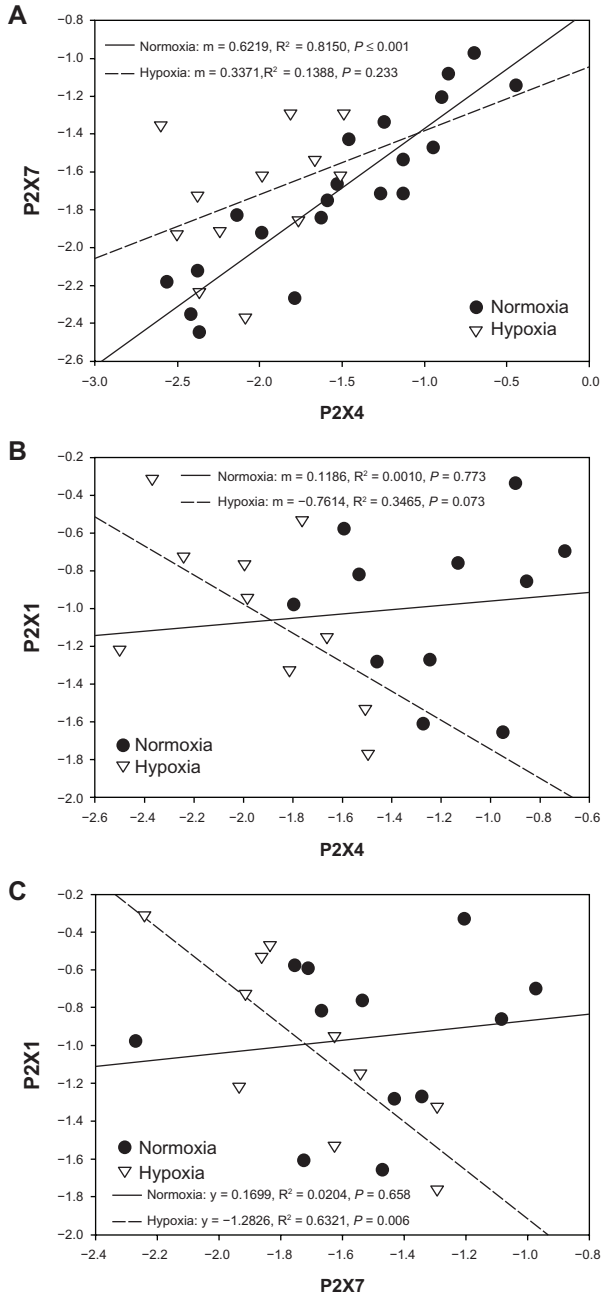

Figure 4 The correlation between P2X4- and P2X7-receptor messenger RNAs in normoxia is lost in hypoxia when P2X1-receptor expression negatively correlates. Multiple linear regression analyses were performed between P2X receptor data (logarithmically transformed interpolated C_T values) in normoxia and hypoxia. Correlations between (**A**) P2X4 and P2X7, (**B**) P2X1 and P2X4, and (**C**) P2X1 and P2X7 are shown. P2X4 expression significantly correlated with both P2X7 in normoxia, an effect that was lost in hypoxia. In hypoxia, P2X1 negatively correlated with both P2X4 and P2X7.
Abbreviation: RNA, ribonucleic acid.

found that exposure to hypoxia for only 2 hours is sufficient to induce microglial *iNOS* and *IL-6* gene expression, detected the next day, although the effects of hypoxia are gene specific, since not all cytokines are similarly upregulated (ie, *TNF*α). As hypoxia and ATP release are often coincident, and P2X receptors are potent regulators of microglial activities, we also tested the impact of P2X receptor activation on *iNOS*, *IL-6*, and *TNF*α expression in hypoxia. We report here that, while P2X receptor activation in normoxia promotes *iNOS* and *IL-6* gene expression (but not *TNF*α), these effects were nearly abolished by hypoxia. We also investigated if the apparent alteration in P2X receptor signaling by hypoxia was related to shifts in the balance of key microglial BzATP-responsive P2X receptors. Although hypoxia alone had no effect on *P2X1*, *P2X4*, or *P2X7* receptor expression, BzATP-stimulated increases in *P2X4* and *P2X7* receptor expression in normoxia were prevented in hypoxia, similar to BzATP effects on *iNOS* and *IL-6* gene expression. As decreases in P2X receptor gene expression were not observed with hypoxia, P2X receptor signaling is probably altered by hypoxia via yet unknown mechanisms.

One possibility contributing to reduced P2X receptor signaling in hypoxia may be the altered composition of P2X receptor heterotrimers or altered interactions among homotrimers. These effects can conceivably occur relatively quickly (ie, within the 2 hours of hypoxia exposure) due to rapid receptor subunit trafficking to the plasma membrane from intracellular pools. Intracellular pools of P2X4, P2X7, and P2X1 receptors have all been reported to influence ion-channel function.[47–50] However, it should be noted that all gene expression analyses in our study were performed one day (22 hours) after the hypoxic exposure ended, so it is possible that alterations in P2X receptor expression may also have contributed to the observed modulation of inflammatory gene expression by BzATP.

In this study, we focused on the role of P2X1, P2X4, and P2X7 receptor regulation of microglial inflammatory activities for several reasons. First, these receptors are among the most highly expressed in brainstem microglia (rank order abundance in freshly isolated adult brainstem microglia is: P2X4 = P2X7 > P2X1 > P2X3 = P2X5) and BzATP has the highest potency at P2X1 and P2X4 receptors.[27] Second, BzATP has higher potency at P2X2, P2X5, and P2X7 receptors versus P2X3 receptors,[27] P2X2 (and P2X6) mRNAs are undetectable in brainstem microglia, and P2X3 (and P2X5) are the least abundant of the P2X receptors in brainstem microglia. Thus, BzATP is most likely to be acting via P2X1, P2X4, and/or P2X7 receptors in brainstem microglia. Third, P2X4 and P2X7 receptors are the best-characterized purinergic receptor subtypes in microglia.[51,52] Little is known concerning P2X1 receptor function in adult microglia, although P2X1 receptors are present on microglia in the developing rat brain.[53] P2X7 receptors regulate microglial production of cytokines such as interleukin-1β and IL-6[46,54] as well as iNOS,[55] whereas

microglial P2X4 receptors contribute to neuropathic pain.[56] Finally, P2X receptors are homo- or heterotrimeric proteins.[57] P2X4 and P2X7 receptor subunits can form heterotrimers,[58] although the preferred configuration in many tissues[59] and in cultured microglia appears to be homotrimers.[48] Importantly, in cultured microglia there are also interactions between P2X4 and P2X7 homotrimers, suggesting cross talk among these P2X receptors, even if they are not components of the same receptor trimer.[48] As P2X1 and P2X4 subunits can also trimerize,[60] P2X4 subunits may be a common "partner" for P2X1 and P2X7 effects. In our studies, we found no effects of hypoxia or nucleotides on *P2X1* receptor mRNA in normoxia; however, *P2X1* receptor expression in hypoxia was negatively correlated with the P2X receptors. A negative association of P2X1 subunits may therefore reflect a complex shift in either the composition of P2X4 and/or P2X7 trimers, and/or altered signaling interactions between these homomeric receptors. P2X receptor trimer composition, or between receptor interactions, have not been evaluated in hypoxia in any system. Thus, it is not yet known if alterations in these parameters contribute to the impaired ability of BzATP to signal to inflammatory genes during hypoxia.

To study the effects of exogenously administered nucleotides on microglial responses, we injected BzATP directly into the cisterna magna. Although the half-life of BzATP in vivo is unknown, this route of administration minimizes tissue trauma caused by direct injection into the brainstem parenchyma, while also maximizing exposure of brainstem cells to high nucleotide levels, the CNS region of interest in these studies. Interestingly, in pure microglial cultures in vitro, we found that between 1 and 8 hours of hypoxia ranging from 1%–15% followed by re-oxygenation for 16–23 hours failed to induce any of the inflammatory genes assessed here (data not shown), suggesting that additional CNS cell types are likely necessary to recapitulate full microglial in vivo responses. This idea is consistent with the brainstem literature indicating an important role of astrocytes in sensing and responding to ATP during hypoxia[61,62] to modulate respiratory rhythm generation and the hypoxic ventilatory response,[22,63–66] key physiologic functions of the brainstem. It is also important to mention that we consider the effects on microglial gene expression observed here to be the result of hypoxia. However, because the animals controlled their own CO_2 during the hypoxic exposures, they also became hypocapnic. Thus, at this time, we cannot rule out the possibility that hypocapnia or a combination of both hypoxia and hypocapnia play a role in the observed effects.

Identifying the individual contributions of P2X4 receptors to microglial activities in hypoxia in vivo is challenging because selective agonists or antagonists for P2X4 receptors are not available. Most general P2 receptor antagonists have no affinity for P2X4. 2′,3′-O-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) has some ability to antagonize P2X4 receptors at high doses, but it has even higher potency at P2X7 receptors,[27] making it difficult to pharmacologically distinguish P2X4 receptor-specific effects in cells such as microglia, where both receptor subtypes are highly expressed and are probably playing opposing roles. Moreover, antagonists such as Iso-Pyridoxalphosphate-6-azophenyl-2′,5′-disulfonic acid (iso-PPADS) that lack the P2X7 receptor activities, have effects at *P2X1* receptors which are also highly expressed in brainstem microglia and which we hypothesize play a role in responses to hypoxia. Due mostly to the lack of highly selective P2X receptor ligands, very little is known about P2X1 receptor activities in microglia in any situation, and little is known about P2X4 receptors in regulating microglial inflammatory gene expression in vivo. Indeed, the best-studied role of P2X4 receptors is their function in tactile allodynia and neuropathic pain.[67,68] Importantly, both P2X4 and P2X7 receptor protein levels are upregulated in microglia following ischemia,[69–71], but no studies to our knowledge have evaluated P2X4 receptor levels in adult microglia after exposure to hypoxia alone[72] or what the functional correlate is of P2X4 upregulation.

There are advantages and disadvantages to the immunomagnetic microglial isolation method used here. The major strength is that all experimental manipulations are performed in vivo, and microglia are rapidly isolated and analyzed. The ability to directly assess microglial gene expression in freshly isolated cells is critical for making the most accurate conclusions about microglia. Typically, changes in microglial morphology are coupled with the presence of inflammatory mRNAs in whole tissue homogenates, the source of which is then ascribed to microglia. These conclusions may or may not be correct. However, the ability to perform these direct and microglia-specific analyses also comes at a cost. Microglia only comprise between 5% and 10% of all CNS cells, and unlike in the rat cortex where 10% of the cells are microglia, only about ~5% of brainstem cells are microglia (data not shown). Therefore, performing protein analyses by Western blots or enzyme-linked immunosorbent assays is not feasible due to limited sample availability. Immunohistochemical analyses are not appropriate for investigations of secreted molecules such as cytokines.

In this study, we were interested in identifying changes in mRNA levels for inflammatory genes induced by hypoxia and/or P2X receptor activation in microglia. In addition to the well-established roles of these inflammatory molecules in neurotoxicity as already outlined, we also chose to evaluate the expression of these particular genes because each one is inducible in microglia. They are regulated primarily at the level of transcription in response to common inflammatory stimuli such as lipopolysaccharide.[73–76] Thus, although concomitant increases in protein levels were not specifically investigated here, they are likely to occur. The transcriptional regulation of these genes is complex, and depending on the inflammatory stimulus used, may involve the transcriptional activities of several transcription factors including nuclear factor of kappa B, activator protein 1, early growth response factors, and cAMP response element-binding protein.[46,77–79] Important with regard to P2X receptor signaling in hypoxia is the fact that all of these transcription factors can be independently regulated by hypoxia[80–82] and P2X receptor activation.[46,83–85] However, because both stimuli individually increase the activation of these transcription factors, we would predict that simultaneous activation of P2X receptors in hypoxia would result in augmented inflammatory gene expression if these transcription factors were involved, rather than the inhibition that we observed. Thus, it seems likely that the activation of a transcriptional repressor or the recruitment of a transcriptional co-repressor in the context of hypoxia may occur in response to P2X receptor activation. To our knowledge, no information exists on specific activation or recruitment of transcriptional repressors or co-repressors by P2X receptors.

Conclusion

The study reported here has shown that hypoxia prevents P2X receptor signaling to inflammatory gene expression in adult brainstem microglia in vivo. Detection of extracellular nucleotides by P2X receptors is likely one mechanism through which microglia sense disturbances in cellular homeostasis in the CNS. That P2X receptor activation promotes inflammatory gene expression in microglia in normoxia suggests that, in pathologic situations when ATP is abundant, microglial inflammatory activities may contribute to neural injury. We suspect that downregulated P2X receptor signaling during hypoxia may be an adaptive measure used by microglia to prevent exaggerated inflammatory responses to the combined stimuli of increased extracellular nucleotides and hypoxia. This adaptation may represent a neuroprotective or anti-inflammatory function of microglia to mitigate hypoxic injury to neurons in this critical CNS region. These results indicate that microglia inherently adapt in hypoxia to mitigate their responsiveness to high concentrations of extracellular ATP when present. Indeed, if this is a generalized mechanism in microglial responses, this pathway could also be detrimental by inducing microglial quiescence during pathological periods of cellular dysregulation when microglial inflammatory activities would be beneficial. For example, the centers of growing tumors are hypoxic and contain high levels of extracellular ATP due to insufficient angiogenesis/vascularization[86] and cell death, respectively.[87,88] The desired microglial response in this situation is an increased production of pro-inflammatory/anti-tumorigenic molecules. However, microglia located directly in the tumor microenvironment are generally immunologically suppressed (reviewed in Watters et al),[89] and underlying mechanisms of this may involve similar alterations in P2X receptor signaling in hypoxia. Thus, identifying mechanisms that regulate microglial responses to the combination of hypoxia and extracellular nucleotides will provide new insights and understanding into microglial regulation and identify new therapeutic targets that can be used to manipulate microglial activities in various CNS pathologies.

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Disclosure

The authors declare no conflicts of interest in this work.

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