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Oxidative Stress Induces Release of 2'-AMP from Microglia

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Abstract

Background: Microglia metabolize exogenous 2'-AMP and 3'-AMP (non-canonical nucleotides) to adenosine and exogenous 2'-AMP and 3'-AMP (via conversion to adenosine) inhibit the production of inflammatory cytokines by microglia. This suggests that if microglia release endogenous 2'-AMP and/or 3'-AMP in response to injurious stimuli, this would complete an autocrine/paracrine mechanism that attenuates the over-activation of microglia during brain injury. Here we investigated in microglia (and for comparison astrocytes and neurons) the effects of injurious stimuli on extracellular and intracellular levels of 2',3'-cAMP (2'-AMP and 3'-AMP precursor), 2'-AMP, and 3'-AMP.

Methods: Experiments were conducted in primary cultures of rat microglia, astrocytes, and neurons. Cells were exposed to oxygen/glucose deprivation, iodoacetate plus 2,4-dinitrophenol (metabolic inhibitors), glutamate, or H₂O₂ for one hour, and extracellular and intracellular 2',3'-cAMP, 2'-AMP, and 3'-AMP were measured by UPLC-MS/MS.

Key Results: In microglia, H₂O₂ increased extracellular levels of 2'-AMP, but not 3'-AMP, by ~16-fold (from 0.17±0.11 to 2.78±0.27 ng/10⁶ cells; n=13; mean ± SEM; P<0.000005). H₂O₂ also induced oxidative changes in cellular proteins as detected by an increased number of carbonyl groups in protein side chains. In contrast, oxygen/glucose deprivation, metabolic inhibitors, or glutamate had no effect on either extracellular 2'-AMP or 3'-AMP levels. In astrocytes and neurons, none of the injurious stimuli increased extracellular 2'-AMP or 3'-AMP.

Conclusions: Oxidative stress (but not oxygen/glucose deprivation, energy deprivation, or excitotoxicity) induces microglia (but not astrocytes or neurons) to release 2'-AMP, but not 3'-

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AUTHORS' CONTRIBUTIONS

TCJ, SEK, and EKJ designed the experiments, analyzed the data, and prepared the first draft of the manuscript. SEK cultured the cells, applied treatments, and harvested samples. EKJ provided the UPLC-MS/MS measurements. PMK provided key revisions of the manuscript and interpretation. PMK and EKJ provided funding.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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AMP. The 2',3'-cAMP/2'-AMP/adenosine pathway mechanism may serve to prevent over-activation of microglia in response to oxidative stress.

Keywords

2',3'-cAMP; 2'-AMP; 3'-AMP; microglia; astrocytes; neurons; oxidative stress; CNPase

1. INTRODUCTION

Microglia, the primary innate immune cells in the brain, constantly survey brain tissues for signs of infection or injury, and in response to danger or damage signals become activated (Kabba et al., 2018; Yin et al., 2017). The *regulated* activation of microglia is neuroprotective and contributes to the killing of invading pathogens and to clean-up and repair operations (Du et al., 2017; Ulland et al., 2015). However, as with most immune cells, *uncontrolled* activation of microglia can be detrimental (Loane et al., 2014; Loane and Kumar, 2016; Witcher et al., 2015). The adverse effects of over-activated microglia are in part mediated by excessive release of pro-inflammatory cytokines (Han et al., 2014; Matsui and Kakeda, 2008) and over-production of oxidative-stress-inducing reactive oxygen species (Block et al., 2007; Loane et al., 2014). Therefore, once microglia are activated, negative feedback mechanisms are essential to prevent microglia from becoming too pro-inflammatory and too pro-oxidative stress-inducing; however, the biochemical nature of these restraints remain unclear.

Brain trauma causes the release of 2',3'-cAMP into the interstitial compartment (Verrier et al., 2012). 2',3'-cAMP is a positional isomer of 3',5'-cAMP that is formed when RNA degradation is accelerated (Jackson et al., 2009). Once produced, 2',3'-cAMP can be converted to either 2'-AMP by the enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) or to 3'-AMP by uncharacterized enzyme(s) with 2',3'-cyclic nucleotide 2'-phosphodiesterase activity (Jackson, 2017; Rao et al., 2010; Verrier et al., 2012; Verrier et al., 2013) or possibly by non-enzymatic mechanisms (Rao et al., 2010). Subsequently, both 2'-AMP and 3'-AMP are converted to adenosine (Jackson et al., 2009; Jackson et al., 2010a; Jackson et al., 2011a; Jackson et al., 2011b; Jackson and Gillespie, 2012; Verrier et al., 2011; Verrier et al., 2013), which can stimulate G-protein coupled adenosine receptors.

Previously we have proposed that the 2',3'-cAMP/2'-AMP/adenosine pathway or the 2',3'-cAMP/3'-AMP/adenosine pathway may be biochemical mechanisms that constrain the activation of microglia. Consistent with this hypothesis, our past studies demonstrate that primary microglia, astrocytes, and neurons convert 2',3'-cAMP to 2'-AMP and 3'-AMP, and metabolize 2'-AMP and 3'-AMP to adenosine (Verrier et al., 2011; Verrier et al., 2013). Thus autocrine or paracrine 2',3'-cAMP/2'-AMP/adenosine or 2',3'-cAMP/3'-AMP/adenosine pathways could provide extracellular adenosine that regulates microglia. Our past studies also show that exogenous 2',3'-cAMP, 2'-AMP, 3'-AMP, and adenosine inhibit the release of inflammatory cytokines, particularly TNF- α and CXCL10, by microglia (Newell et al., 2015). Moreover, these anti-inflammatory responses are mimicked by the selective A_{2A}-receptor agonist CGS21680 and are blocked by antagonism of adenosine receptors (Newell et al., 2015). Finally, our past studies demonstrate that traumatic brain injury (TBI)

induces a greater microglia response (20% to 50% increase in microglia in the in the ipsilateral cortex, CA3, and thalamus, and contralateral cortex, CA1, and thalamus) in A_1 receptor control mechanisms that limit microglia migration or proliferation. Taken together, thus far the experimental evidence supports the concept that 2'-AMP and/or 3'-AMP are part of a feedback system that restrains the activation of microglia.

There is a knowledge gap, however, that must be addressed to corroborate the role of 2'-AMP and/or 3'-AMP in regulating microglia. Specifically, whether injurious stimuli induce brain cells to release endogenous 2'-AMP and/or 3'-AMP is unknown and the goal of the current study was to address this knowledge gap. To achieve this objective, we cultured rat microglia, astrocytes, and neurons and provoked them by restricting substrate availability (in this case, oxygen and glucose deprivation), blocking glycolysis and oxidative phosphorylation (with iodoacetate plus 2,4-dinitrophenol), inducing excitotoxicity (with glutamate), or increasing oxidative stress (with H_2O_2). Both extracellular and intracellular levels of 2',3'-cAMP, 2'-AMP, and 3'-AMP were measured using ultra-performance liquid chromatography combined with tandem mass spectrometry (UPLC-MS/MS).

2. RESULTS

2.1. Study 1: Effects of Injurious Stimuli on 2',3'-cAMP, 3'-AMP, and 2'-AMP in Rat Primary Microglia.

Rat primary microglia were treated with either: 1) control medium; 2) 40 μM of H_2O_2 to induce oxidative stress; 3) 50 μM of iodoacetate (inhibits glycolysis) plus 50 μM of 2,4-dinitrophenol (inhibits oxidative phosphorylation) to pharmacologically block energy-producing pathways; 4) medium lacking glucose and equilibrated at 0.2% oxygen to deprive cells of energy generating substrates; or 5) 10 μM of glutamate to induce excitotoxicity. After 1 hour, medium was collected (extracellular compartment) and cells were extracted (intracellular compartment) and these samples were subjected to UPLC-MS/MS for quantification of intracellular and extracellular 2',3'-cAMP, 3'-AMP, and 2'-AMP.

In **Study 1** we included 20 pairs of 35-mm culture plates of microglia (500,000 cells per plate; 40 plates total). An experimental unit ($n=1$) was a pair of plates consisting of 1 plate of control cells and 1 corresponding plate of injured cells in which each pair was derived from a unique batch of day 1-2 postnatal rat pups ($n=5$ biological replicates/group). In this experimental design, one of each pair was a side-by-side control and the other member of the pair was treated with the indicated injurious stimulus (random assignment).

Figure 1 summarizes the effects of H_2O_2 on extracellular and intracellular levels of 2',3'-cAMP, 3'-AMP, and 2'-AMP in microglia. In microglia, H_2O_2 did not significantly affect extracellular levels of 2',3'-cAMP (Figure 1A), intracellular levels of 2',3'-cAMP (Figure 1B), extracellular levels of 3'-AMP (Figure 1C) intracellular levels of 3'-cAMP (Figure 1D), or intracellular levels of 2'-AMP (Figure 1F). However, H_2O_2 induced a striking increase in extracellular levels of 2'-AMP (from 0.23 ± 0.23 to 2.52 ± 0.22 ng per million microglia; $P=0.0043$, 2-tailed, paired Student's *t*-test; Figure 1E). In microglia treated with either iodoacetate plus 2,4-dinitrophenol, oxygen and glucose deprivation, or glutamate, levels of

intracellular or extracellular 2',3'-cAMP, 3'-AMP, or 2'-AMP were less than the assay detection limit (0.05 ng/ml), indicating no increase in any of these purines with any of these treatments in either the intracellular or extracellular compartments.

2.2. Study 2: Confirmation of the Effects of H₂O₂ on 2',3'-cAMP, 3'-AMP, and 2'-AMP in Rat Primary Microglia.

Study 1 examined the effects of four different injurious stimuli on three different purines in two different compartments (extracellular and intracellular). Therefore, it is conceivable that the large effect of H₂O₂ on extracellular 2'-AMP was due to random chance. The purpose of **Study 2** was to challenge the robustness and reproducibility of our positive findings in **Study 1** with regard to the effects of H₂O₂ on extracellular levels of 2'-AMP in microglia. To achieve this, in **Study 2** (which was performed using different timed-pregnant rats) we focused our experiments on the effects of H₂O₂ on extracellular and intracellular levels of 2',3'-cAMP, 3'-AMP, and 2'-AMP. In addition, we increased the sample size to 8 pairs of matching plates of microglia (n=8 biological replicates/group). As shown in Figure 2, the results obtained from **Study 2** were very similar to the results observed in **Study 1**. H₂O₂ did not significantly affect extracellular levels of 2',3'-cAMP (Figure 2A), intracellular levels of 2',3'-cAMP (Figure 2B), extracellular levels of 3'-AMP (Figure 2C) intracellular levels of 3'-cAMP (Figure 2D), or intracellular levels of 2'-AMP (Figure 2F), yet significantly increased extracellular levels of 2'-AMP (from 0.14 ± 0.10 to 2.94 ± 0.42 ng per million microglia; $P=0.0007$, 2-tailed, paired Student's t-test; Figure 2E). Since the protocols for **Study 1** and **Study 2** were identical and the results obtained in **Study 1** and **Study 2** were similar, we combined all 13 experimental pairs (Control versus H₂O₂-treated) to yield an even more robust estimate of the effects of H₂O₂ on extracellular 2'-AMP levels. As shown in Figure 3A, taken together our experiments indicate that H₂O₂ increased extracellular 2'-AMP levels from 0.17 ± 0.10 to 2.78 ± 0.27 ng per million microglia ($P=0.000005$, 2-tailed, paired Student's t-test).

We also combined the data in Figures 1 and 2 for each variable in panels **A**, **B**, **C**, **D**, and **F** to give n=13 for control cells and n=13 for H₂O₂-treated cells and subjected these combined data to a statistical analysis (2-tailed paired Student's t-test). The results were not-significant for any of the 5 variables. Next, using the standard deviation of the differences between control and H₂O₂-treated cells for these variables, we determined the statistical power for detecting an effect that was 1/10th the effect of H₂O₂ on extracellular 2'-AMP (the variable that changed substantially). Using a p-value of 0.05, a sample size of n=13, and standard deviations directly determined by our experiments, we calculated that the power to see 1/10th the effect of H₂O₂ on extracellular 2'-AMP was greater than 90% for all 5 variables. Therefore, we conclude that with regard to the variables listed in panels **A**, **B**, **C**, **D**, and **F**, if there was an effect of H₂O₂, the effect was less than 1/10th of that for extracellular 2'-AMP and therefore was unlikely to be of biological significance.

In all 26 plates of microglia (i.e., 13 pairs of cells), we also measured by UPLC-MS/MS the extracellular levels of adenosine. As shown in Figure 3B, there was a highly significant linear correlation between extracellular levels of 2'-AMP and extracellular levels of

adenosine ($P < 0.0001$), and extracellular 2'-AMP explained approximately 50% of the variability in extracellular levels of adenosine ($R^2 = 0.5226$).

Although H_2O_2 is an efficacious oxidizing agent, it is important to confirm that the concentration of H_2O_2 used in the present study induced oxidative changes. Therefore we employed the OxyBlot™ Kit that provides a sensitive immunodetection approach to detect carbonyl groups introduced into proteins by reactions involving oxygen free radicals. This method uses the reagent 2,4-dinitrophenylhydrazine (DPH) to derivatize carbonyl groups in protein side chains to 2,4-dinitrophenylhydrazone, which is detected by western blotting with a primary antibody to the 2,4-dinitrophenylhydrazone moiety. As shown in Figure 4, a positive control provided by the kit gave a clear signal, indicating that the assay system was sensitive. Equal amounts of protein from microglia treated or not with H_2O_2 were processed either in the presence of DPH or without DPH (negative control). As shown, in the presence of DPH, microglia pretreated with H_2O_2 gave a much darker image compared with microglia not treated with H_2O_2 . Importantly, no signal was observed in protein samples processed in the absence of DPH (negative control). These experiments confirm that 40 μM of H_2O_2 indeed induced oxidative stress.

2.3. Study 3: Effects of Injurious Stimuli on 2',3'-cAMP, 3'-AMP, and 2'-AMP in Rat Primary Astrocytes and Neurons.

We also examined the effects of H_2O_2 , iodoacetate plus 2,4-dinitrophenol, oxygen and glucose deprivation, and glutamate on intracellular and extracellular levels of 2',3'-cAMP, 3'-AMP, and 2'-AMP in astrocytes and neurons. In this regard, the study design was identical to that described for microglia. For astrocytes we examined the effects of H_2O_2 , iodoacetate plus 2,4-dinitrophenol, oxygen and glucose deprivation, and glutamate in 7, 7, 8, and 4 pairs of culture plates, respectively; and for neurons, the sample size was 5, 10, 4, and 5 pairs of culture plates, respectively.

In both astrocytes and neurons, the intracellular and extracellular levels of 2',3'-cAMP, 3'-AMP, or 2'-AMP were below or near the detection limit of our assay system either in the absence or presence of the injurious stimuli (Table 1 in online supplement), and, with one exception, none of the injurious stimuli increased extracellular or intracellular levels of 2', 3'-cAMP, 3'-AMP, or 2'-AMP. The one exception was that in astrocytes, intracellular levels of 2'-AMP were increased slightly, but significantly, by treatment with iodoacetate plus 2,4-dinitrophenol (from less than detection limit to 0.07 ± 0.02 ng per million cells; $P = 0.0103$).

3. DISCUSSION

This is the first study to examine whether injurious stimuli trigger microglia to produce and release the non-canonical nucleotides 3'-AMP and 2'-AMP. A novel and important finding of the present study is that when microglia are exposed to oxidative stress they produce 2'-AMP. The production of 2'-AMP by microglia in response to oxidative stress results in a remarkable ~16-fold increase in the extracellular levels of 2'-AMP. Because microglia can metabolize extracellular 2'-AMP to adenosine (Verrier et al., 2011) and because extracellular 2'-AMP, via its metabolism to adenosine, inhibits the ability of microglia to manufacture and release cytokines (Newell et al., 2015), the finding that oxidative stress has

this effect on microglia suggests that the 2',3'-cAMP/2'-AMP/adenosine pathway may function to restrain the activation of microglia and thereby protect against brain injuries that are accompanied by oxidative stress.

In the present study, microglia were treated with 40 μ M of H₂O₂ for 1 hour to induce oxidative stress. A critical issue is whether this treatment paradigm was sufficient to cause oxidative stress. Our OxyBlot™ experiments to detect carbonyl moieties in amino acid side chains indeed confirmed that 40 μ M of H₂O₂ for 1 hour did trigger oxidative stress. Another important issue is whether 40 μ M of H₂O₂ for 1 hour ruptured cell membranes, thus releasing 2'-AMP. Very recently, we published that treatment of microglia with 40 μ M of H₂O₂ for 1 hour does not release lactic acid dehydrogenase (Jackson et al., 2017), a finding that rules out membrane rupture as the mechanism by which oxidative stress causes 2'-AMP release from microglia.

It is important to note that microglia express CNPase (Wu et al., 2006), that CNPase expression is increased in activated microglia (Yang et al., 2014), and that knockdown of CNPase in microglia results in over-production of inflammatory mediators (Yang et al., 2014). Inasmuch as CNPase is the dominant enzyme responsible for metabolizing 2',3'-cAMP to 2'-AMP (Verrier et al., 2012), these previous findings are consistent with our hypothesis that the 2',3'-cAMP/2'-AMP/adenosine pathway functions to restrain the activation of microglia.

Also consistent with our hypothesis that the 2',3'-cAMP/2'-AMP/adenosine pathway is neuroprotective are reports that similar levels of brain injury yield worse outcomes in CNPase knockout (CNPase^{-/-}), compared with wild-type (CNPase^{+/+}), mice. For example, our previous work demonstrates that controlled cortical impact (CCI) in CNPase^{+/+} mice does not increase brain interstitial levels of 2',3'-cAMP, but does increase by several fold brain interstitial levels of 2'-AMP (Verrier et al., 2012). This indicates that some brain cell types make 2',3'-cAMP in response to injury; yet most, if not all, of the 2',3'-cAMP is rapidly converted to 2'-AMP. Indeed, in CNPase^{-/-} mice that cannot convert 2',3'-cAMP to 2'-AMP, CCI induces the accumulation of brain interstitial 2',3'-cAMP but does not increase brain interstitial 2'-AMP (Verrier et al., 2012). This lack of conversion of 2',3'-cAMP to 2'-AMP is accompanied by a reduced brain interstitial level of adenosine and a worse histological outcome (Verrier et al., 2012). These in vivo results are in agreement with the current findings in primary cultures of rat microglia. As in vivo, rather than accumulating 2',3'-cAMP, microglia cultures generate extracellular 2'-AMP thus indicating rapid conversion of 2',3'-cAMP to 2'-AMP. Whether this metabolism of 2',3'-cAMP to 2'-AMP occurs intracellular, extracellular, or both remains an open question. Also in agreement with previous in vivo results, in microglia cultures exposed to oxidative stress, extracellular adenosine levels are highly correlated with extracellular 2'-AMP concentrations. These findings further support the concept that extracellular 2'-AMP is converted to neuroprotective adenosine.

Also relevant to and consistent with the current findings are the important results reported by Wieser and colleagues (Wieser et al., 2013). In a landmark study, these investigators activated microglia in the brains of CNPase^{-/-} and CNPase^{+/+} postnatal day 28 mice pups

by applying a mild cryolesion to the right parietal cortex. Three months later, this minor brain injury caused significant microgliosis, axon degeneration, and deterioration of working memory. Our findings are consistent with the results of Wieser and colleagues in that both studies suggest an important connection between the 2',3'-cAMP/2'-AMP/adenosine pathway and neuroinflammation.

A notable feature of the present findings is that unlike microglia, astrocytes and neurons do not respond to oxidative stress by increasing extracellular levels of 2'-AMP. Primary microglia are difficult to culture, so the densities/numbers of microglia were much less than that for neurons and astrocytes. The microglia cultures contained 0.5 million cells, the astrocyte cultures contained 1.8 million cells, and the neuron cultures contained 6 million cells. Therefore, the fact that we could readily detect 2'-AMP in microglia, but not in astrocytes or neurons, was not due to lower densities/numbers of astrocytes or neurons.

A recent study by Ishihara and coworkers (Ishihara et al., 2015) demonstrates that lipopolysaccharide induces a multi-fold increase in superoxide dismutase 2 mRNA and protein in microglia, but not astrocytes or neurons. Since superoxide dismutase converts superoxide anion to H₂O₂, microglia, but not astrocytes or neurons, are programmed to produce the 2'-AMP-stimulating oxidant H₂O₂. Hence microglia appear better equipped to generate H₂O₂ and respond to H₂O₂ by producing 2'-AMP.

Along with the reproducibility of our results, another factor that adds credence to the findings of the present study is the specificity of 2'-AMP production. In this regard, 2'-AMP production is specific with respect to cell type (microglia produce 2'-AMP, but not astrocytes or neurons) and injurious stimulus (oxidative stress stimulates 2'-AMP production, but not excitotoxicity, metabolic inhibitors, or oxygen and glucose deprivation). Also, 2'-AMP production is specific with respect to type of non-canonical nucleotide produced (2'-AMP, but not 3'-AMP). As mentioned, CNPase (a well-characterized mammalian enzyme) converts 2',3'-cAMP to 2'-AMP; but because of steric exclusion of the P-O2' bond from the active site, CNPase does not produce 3'-AMP (Rao et al., 2010). Rao et al. have identified several non-mammalian enzymes that metabolize 2',3'-cAMP to 3'-AMP (Rao et al., 2010); however, currently the mammalian enzymes, if any, that produce 3'-AMP are unknown. Indeed, because the P-O2' bond, compared with the P-O3' bond, of 2',3'-cAMP is weaker and has a lower activation energy (Rao et al., 2010), 2',3'-cAMP is susceptible to non-enzymatic hydrolysis to 3'-AMP and this may be the primary mechanism of 3'-AMP formation. The specificity of 2'-AMP production over 3'-AMP production observed in the current study suggests that before 2',3'-cAMP can be broken down to 3'-AMP non-enzymatically, CNPase converts this non-canonical cyclic nucleotide to 2'-AMP. The rapid conversion of 2',3'-cAMP to 2'-AMP by microglia may be necessary to prevent apoptosis of microglia because as shown by Azarashvili and coworkers (Azarashvili et al., 2009; Azarashvili et al., 2010) and discussed by Raasakka and Kursula (Raasakka and Kursula, 2014), 2',3'-cAMP promotes a pro-apoptotic event, namely mitochondrial permeability transition.

Primary microglia are labor intensive to culture, and culturing primary microglia requires a substantial investment in personnel time, reagents, and animal costs; and they cannot be

passed. For this reason, many investigators use immortalized microglia cells, such as the BV-2 cell line, as a model system for microglia. However, immortalized BV-2 cells may not recapitulate the phenotype of authentic microglia (Horvath et al., 2008). A major strength of the current investigation is that we used primary microglia.

Ideally, it would be informative to examine the effects of oxidative stress on 2'-AMP production in microglia obtained from CNPase^{-/-} animals. In order to do this, however, one must have large litters of homozygous knockout animals. Currently, we have a colony of CNPase knockout mice. However, despite many attempts we find that breeding CNPase^{-/-} males with CNPase^{-/-} females very rarely produces offspring, thus precluding doing experiments with microglia from CNPase^{-/-} mice. It may be possible, however, to accomplish this using CNPase knockout rats and we are pursuing that line of research. Whether CNPase knockout rats will be viable and fertile is presently unknown.

4. CONCLUSIONS

In summary, oxidative stress triggers microglia to produce the non-canonical nucleotide 2'-AMP. Because we have previously shown that 2'-AMP inhibits the release of inflammatory cytokines (e.g., TNF- α and CXCL10) by microglia, we propose the hypothesis that the 2', 3'-cAM/2'-AMP/adenosine pathway functions to limit the activation of microglia during injuries that produce oxidative stress. Our conclusions imply that individuals deficient in this pathway would be at risk of worse outcomes following brain injury.

5. EXPERIMENTAL PROCEDURE

5.1. Animals.

Gravid Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA). The Institutional Animal Care and Use Committee of the University of Pittsburgh approved all animal procedures, which were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

5.2. Microglial Cultures.

Primary microglial cultures were generated from postnatal (day 1-2) male and female Sprague-Dawley rat pups as described by Ni and Aschner (Ni and Aschner, 2010), but with modifications as described by us previously (Jackson et al., 2017). Briefly, brains were harvested, trypsinized, triturated, seeded onto 225-cm² flasks, and cultured in DMEM/F12/10% fetal bovine serum (FBS) containing 100 U/ml of penicillin and 100 μ g/ml of streptomycin. After 1 week, cells were provided a complete exchange of medium every 2-3 days for 2 weeks. After the final complete medium exchange, flasks were gently shaken for 8 minutes, which resulted in dissociation of microglia into the medium. The collected medium was centrifuged (5 minutes at 200 g and 4°C), and microglia were plated onto 35-mm poly-D-lysinecoated culture plates (500,000 cells per plate). Cells were counted at the time of plating (microglia do not proliferate in cell culture) with a Nexcelom Cellometer Auto T4 cell counter (Nexcelom Bioscience: Lawrence, MA). To avoid astrocyte contamination, experiments were performed the day after plating. We have previously

validated using western blotting and immunofluorescence that this protocol yields highly enriched microglia cultures (Jackson et al., 2017).

5.3. Astrocyte Cultures.

Astrocytes were obtained as described by us (Verrier et al., 2011). In brief, brains that were obtained from male and female Sprague-Dawley rat pups at postnatal day 1–2 were trypsinized, triturated, and seeded onto 75 cm² tissue culture flasks. Cells were grown to 90–95% confluence in the same medium as described for microglia. Cells were propagated to purity by several passages, and experiments were performed in 10-cm poly-D-lysine-coated culture plates. Cells were plated at 200,000 cells per plate and allowed to proliferate for 1 week, at which time there were 1,800,000 cells per plate (as assessed by a Nexcelom Cellometer Auto T4 cell counter). We have previously validated using western blotting and immunofluorescence that this protocol yields highly enriched astrocyte cultures (Jackson et al., 2017).

5.4. Neuronal Cultures.

Neurons were cultured from Sprague-Dawley rat embryos (E16–E18) using a method previously developed by us (Jackson et al., 2010b). In brief, male and female embryonic brains were harvested, trypsinized, triturated, and seeded (6,000,000 cells per plate) onto 10-cm poly-D-lysine-coated culture plates (BD Biosciences, San Jose, CA). Cells were counted at the time of plating (neurons do not divide in cell culture) with a Nexcelom Cellometer Auto T4 cell counter. Neurons were grown in Neurobasal medium (Life Technologies, Grand Island, NY) containing B27 supplement, 0.5 mM glutamine, 100 U/ml of penicillin, and 100 µg/ml streptomycin, and maintained by one-half medium exchange on day in vitro 3 and 5 (DIV 3 and DIV 5). Experiments were conducted on day in vitro 6 (DIV 6) because cultures are > 90% pure neurons at that time point (Brewer et al., 1993; Lin et al., 2002) and are less likely to be contaminated by glia cells. We have previously validated using western blotting and immunofluorescence that this protocol yields highly enriched neuronal cultures (Jackson et al., 2017).

5.5. Metabolic Inhibitor Injury.

On the day of the experiment, growth medium was aspirated off of the cells and pre-warmed control medium [Dulbecco's phosphate-buffered saline (DPBS), Gibco #14287] or pre-warmed injury medium [control medium plus 50 µM iodoacetate (IA) and 50 µM 2,4-dinitrophenol (DNP); IA and DNP were from Sigma-Aldrich (St. Louis, MO)] was added to the plates (3 ml of medium for 10-cm plate experiments; 1 ml of medium for 35-mm plate experiments). The concentrations of IA and DNP were selected to only partially (approximately 50%) block the glycolytic pathway (Cardenas-Rodriguez et al., 2009) and oxidative phosphorylation (Livnat et al., 1993), respectively, in order to mimic partial metabolic failure that characterizes brain injury (Jalloh et al., 2015). After 1 hour of incubation (37°C/5% CO₂ incubator), DPBS (i.e., extracellular compartment) was collected, placed in boiling water for 90 seconds so as to inactivate any enzymes, and stored at –80°C. The cells (intracellular compartment) were processed as described below.

5.6. H₂O₂ Injury.

Culture medium was removed and replaced with either pre-warmed balance salt solution (BSS) (NaCl, 137 mM; KCl, 5.36 mM; CaCl₂, 1.26 mM; MgCl₂, 0.49 mM; MgSO₄, 0.41 mM; HEPES, 10 mM; KH₂PO₄, 0.44 mM; NaHCO₃, 4.17 mM; Na₂HPO₄, 0.34 mM; D-glucose, 5 mM; pH 7.4) or pre-warmed injury BSS (control BSS containing 40 µM of H₂O₂). The concentration of H₂O₂ used in this study (40 µM) is similar to that achieved in brain tissue following brain ischemia-reperfusion injury (Lei et al., 1997) and induces neuronal cell death (Jackson et al., 2013) and oxidative stress (Ma et al., 2014) in DIV6 cortical neurons. For experiments in 10-cm plates, 3 ml of control or injury medium was used; and for 35-mm plates, 1 ml of control or injury medium was applied. After 1 hour of incubation (37°C/5% CO₂ incubator), BSS (i.e., extracellular compartment) was collected, placed in boiling water for 90 seconds so as to inactivate any enzymes, and stored at -80°C. The cells (intracellular compartment) were processed as described below.

5.7. Glutamate Injury.

Culture medium was removed, and either pre-warmed DPBS (Gibco #14287) or pre-warmed injury DPBS [control DPBS supplemented with 10 µM L-glutamate (Sigma-Aldrich)] was applied to the cultures (3 ml of medium for 10-cm plates and 1 ml of medium for 35-mm plates). The concentration of glutamate used in this study (10 µM) is similar to that achieved following severe brain injury (Timofeev et al., 2011). Plates were incubated for 1 hour (37°C/5% CO₂ incubator), and DPBS (extracellular compartment) was collected and placed in boiling water for 90 seconds so as to inactivate any enzymes, and stored at -80°C. The cells (intracellular compartment) were processed as described below.

5.8. Oxygen-Glucose Deprivation (OGD) Injury.

One day before experiments, DPBS (Gibco #14287) and injury DPBS (Gibco #14040 with no glucose and supplemented with 0.327 mM of sodium pyruvate) were pre-equilibrated by placing them inside a normoxic incubator or hypoxic incubator, respectively. The internal gas mixture for the normoxic incubator was 5% CO₂ and 95% room air. The internal gas mixture of the hypoxic incubator was 5% CO₂ with O₂ levels maintained at 0.2% by constant flushing of the incubator with 100% N₂. We selected 0.2% oxygen with no glucose to mimic brain injury induced by ischemia/anoxia because low blood flow reduces brain tissue levels of oxygen and glucose. Plates were incubated for 1 hour with either DPBS in the normoxic incubator or injury DPBS in the hypoxic incubator. Medium was then collected and placed in boiling water for 90 seconds so as to inactivate any enzymes, and stored at -80°C. The cells (intracellular compartment) were processed as described below.

5.9. Analysis of 2',3'-cAMP, 2'-AMP, and 3'-AMP.

2',3'-cAMP, 2'-AMP, and 3'-AMP in the extracellular (DPBS/BSS) and intracellular (cell extract) compartments were measured by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). After the indicated treatments and incubations, the medium was acquired, and ¹³C₅-2',3'-cAMP (Toronto Research Chemicals, Toronto, Ontario, Canada), ¹³C₅-2'-AMP (13C Molecular, Fayetteville, NC), and ¹³C₅-3'-AMP (13C Molecular) were added directly to the medium to serve as heavy isotope internal standards.

The medium samples were placed in a -80°C ultralow freezer until analyzed by UPLC-MS/MS.

Immediately after collecting the medium, 1 to 3 ml of ice-cold acetonitrile:methanol:water (1:2:2) was added to the cell monolayers. The cells were scraped, and the entire sample was sonicated to extract any intracellular 2',3'-cAMP, 2'-AMP, and 3'-AMP. Next, the samples were heated (60°C water bath for 10 minutes) to denature enzymes that might destroy these purines. At this point, the cell extracts were placed in a -80°C ultralow freezer until further processing. Thawed cell extracts were centrifuged (3000 g for 90 minutes at 4°C). The supernatant was collected and placed in a new tube. The cell pellet was treated with an additional 0.5 to 2 ml of acetonitrile:methanol:water to extract any remaining purines, and the cell pellet was centrifuged and the supernatant was collected and combined with that from the first extraction. Using a sample concentrator, the supernatants were dried, reconstituted in H_2O , passed through a 30-kDa cutoff filter, and $^{13}\text{C}_5$ -2',3'-cAMP, $^{13}\text{C}_5$ -2'-AMP, and $^{13}\text{C}_5$ -3'-AMP were added to the collected H_2O . The recovery of 2',3'-cAMP, 2'-AMP, and 3'-AMP using this procedure was approximately 96% (as assessed by the signal from internal standards added to samples before versus after processing).

2',3'-cAMP, 2'-AMP, and 3'-AMP in medium and cell extracts were quantified using UPLC-MS/MS as previously described by us (Jackson et al., 2009) with modifications. Samples were injected into a reversed-phase liquid chromatography (Waters UPLC BEH C18 column, $1.7\ \mu\text{m}$ beads; $2.1 \times 150\ \text{mm}$; Milford, MA) and assayed using a TSQ Quantum-Ultra mass spectrometer (ThermoFisher Scientific, San Jose, CA) operating in the selected reaction monitoring (SRM) mode with a heated electrospray ionization source. The mobile phase was a linear gradient of 1% acetic acid in water (pH, 3; mobile phase A) and 100% methanol (mobile phase B). The mobile phase was delivered to the column at a flow rate of 300 $\mu\text{L}/\text{minute}$ with a Waters Acquity ultra-performance liquid chromatographic system. The gradient (A/B) was: from 0 to 2 minutes, 99.6%/0.4%; from 2 to 3 minutes, to 98.0%/2.0%; from 3 to 4 minutes, to 85.0%/15.0%; from 4 to 6.5 minutes, to 99.6%/0.4%. Instrument settings were: sample tray temperature, 10°C ; column temperature, 50°C ; ion spray voltage, 4.0 kilovolts; ion transfer tube temperature, 350°C ; source vaporization temperature, 320°C ; Q2 CID gas, argon at 1.5 mTorr; sheath gas, nitrogen at 60 psi; auxiliary gas, nitrogen at 35 psi; Q1/Q3 width, 0.7/0.7 units full-width half-maximum; scan width, 0.6 units; scan time, 0.01 seconds. The following mass transitions were monitored and used for generating standard curves and calculating the amounts of 2',3'-cAMP, 2'-AMP, and 3'-AMP in the samples: $^{13}\text{C}_5$ -2',3'-cAMP ($335 \rightarrow 136\ \text{m/z}$), $^{13}\text{C}_5$ -2'-AMP ($353 \rightarrow 136\ \text{m/z}$), $^{13}\text{C}_5$ -3'-AMP ($353 \rightarrow 136\ \text{m/z}$), 2',3'-cAMP ($330 \rightarrow 136\ \text{m/z}$), 2'-AMP ($348 \rightarrow 136\ \text{m/z}$), and 3'-AMP ($348 \rightarrow 136\ \text{m/z}$). In the microglia experiments, we also measured extracellular levels of adenosine ($368 \rightarrow 136\ \text{m/z}$) using $^{13}\text{C}_{10}$ -adenosine ($376 \rightarrow 141\ \text{m/z}$; Medical Isotopes, Pelham, NH) as the internal standard. 2',3'-cAMP, 2'-AMP, 3'-AMP, and adenosine for standard curves were obtained from Sigma Aldrich.

5.10. Assessment of Oxidative Stress.

In the present study, we used 40 μM of H_2O_2 to induce oxidative stress. To confirm that this concentration of H_2O_2 did induce oxidative stress-induced changes in cellular proteins, we

employed the OxyBlot™ Protein Oxidation Detection Kit (MilliporeSigma, Burlington, MA). In this regard, oxygen free radicals react with proteins and introduce carbonyl groups into protein side chains by a site-specific mechanism. The OxyBlot™ Kit provides a sensitive immunodetection approach to detect these carbonyl groups.

Primary microglial cultures were harvested, plated, and treated with 40 μ M of H₂O₂ for 1 hour as described above. The plating density was increased 4-fold to 2,000,000 cells per plate to increase the protein concentration of the samples. After the 1 hour incubation, control or injury medium was aspirated from the plates and 60 μ l of ice cold RIPA Buffer (ThermoFisher Scientific) supplemented with 1% EDTA and 1% protease/phosphatase inhibitors was added. Microglia were then scraped, collected, sonicated for 20 seconds, centrifuged at 16,000 g for 10 minutes at 4°C, and stored at –80°C for further analysis.

Protein concentrations of microglia homogenates were determined with the use of the Pierce BCA Protein Assay Kit (ThermoFisher Scientific). Samples were diluted by the addition of supplemented RIPA Buffer to a final concentration of 200 ng/ μ l. Oxidative stress was measured with the use of the OxyBlot™ Protein Oxidation Detection Kit. All buffers/reagents were made in accordance with the manufacturer's protocol. Briefly, 5 μ l of sample (1 ng of protein) was used for the derivatization reaction and the negative control. Samples were denatured with the addition of 12% SDS, then 1X 2,4-dinitrophenylhydrazine was added to the derivatized samples and 1X Derivatization Control Solution was added to the negative control samples. After a 15-minute incubation at room temperature, Neutralization Solution was added to all samples.

Prepared samples were then loaded into a 4-15% gradient SDS-PAGE TGX precast gel (BioRad, Hercules, CA) for electrophoresis. After electrophoresis, proteins were transferred to a 0.2 μ m PVDF membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA) that was pre-wet with 95% EtOH and rinsed with nanopure water. The transfer membrane was then incubated in Blocking/Dilution Buffer for 1 hour at room temperature. After 1 hour incubation, 1° Antibody (1:150) was added for 1 hour, rinsed 3X with PBS-T, incubated in 2° Antibody (1:300) for 1 hour, then again rinsed 3X with PBS-T. Pierce ECL-2 Chemiluminescent Reagent (ThermoFisher Scientific) was then added to the membrane for 90 seconds and images were gathered in a dark room.

5.11. Statistical Analysis.

Using the assay system described above, the total amounts of 2',3'-cAMP, 2'-AMP, and 3'-AMP in the medium (extracellular) and cell extracts (intracellular) were calculated and normalized to cell number (ng/10⁶ cells). To ensure non-biased results, the analytical chemist who performed the UPLC-MS/MS assays was blinded to the treatment groups. An experimental unit was defined as a separate batch of cells from a batch of embryos or pups. To reduce variability, we employed a paired experimental design in which each experimental unit was divided into two paired plates of cells. Thus each treated plate had a paired control plate of cells, and assignment as to whether a plate received the indicated injurious treatment or was sham-treated was determined randomly. Thus data were analyzed using a 2-tailed paired Student's t-test (criterion of significance was $p < 0.05$). A power analysis was conducted on variables that did not achieve statistical significance to ascertain the magnitude

of change that could have been detected by the experiments. Statistical analyses were performed using the Number Cruncher Statistical Systems (NCSS 2004 for t-tests and PASS for power analysis, Kaysville, Utah). All values in the text and figures are means and SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- We examined production of non-canonical nucleotides by brain cells.
- In microglia, oxidative stress increased extracellular 2'-AMP by ~16-fold.
- In astrocytes and neurons, oxidative stress did not increase extracellular 2'-AMP.
- In microglia, extracellular 2'-AMP and adenosine were correlated.
- 2'-AMP may regulate microglia via adenosine.

Study 1

Figure 1. Study 1 examined the effects of H₂O₂ on extracellular and intracellular levels of 2',3'-cAMP, 3'-AMP, and 2'-AMP in rat primary microglia.

This study included 5 pairs of plates of primary rat microglia cultured from different postnatal day 1-2 rat pups. For each pair of plates, 1 plate was selected randomly for treatment with H₂O₂ (40 μ mol/L) and the corresponding paired plate was not treated (Control). After 1 hour, and 2',3'-cAMP (panels A and B), 3'-AMP (panels C and D), and 2'-AMP (panels E and F) were measured in the extracellular compartment (medium; panels A, C, and E) or intracellular compartment (cell extracts; panels B, D, and F) by ultra-performance liquid chromatography-tandem mass spectrometry. Results were normalized to cell number. Note that the scale in panel E is extended 10-fold to the range of 0 to 4 ng/10⁶ cells to accommodate the large increase in extracellular 2'-AMP induced by H₂O₂ ($P=0.0043$; 2-tailed, paired Student's t-test). Values indicate means and SEM.

Study 2

Figure 2. Study 2 confirmed that H₂O₂ increases extracellular levels in rat primary microglia. We observed in **Study 1** that H₂O₂ induced a very large (approximately 10-fold) increase in extracellular 2'-AMP. In Study 2, we challenged this finding by repeating the study protocol in its entirety using an even larger sample size. This study included 8 pairs of plates of primary rat microglia cultured from different postnatal day 1-2 rat pups. For each pair of plates, 1 plate was selected randomly for treatment with H₂O₂ (40 μmol/L) and the corresponding paired plate was not treated (Control). After 1 hour, and 2',3'-cAMP (panels A and B), 3'-AMP (panels C and D), and 2'-AMP (panels E and F) were measured in the extracellular compartment (medium; panels A, C, and E) or intracellular compartment (cell extracts; panels B, D, and F) by ultra-performance liquid chromatography-tandem mass spectrometry. Results were normalized to cell number. Note that the scale in panel E is extended 10-fold to the range of 0 to 4 ng/10⁶ cells to accommodate the large increase in extracellular 2'-AMP induced by H₂O₂ ($P=0.0007$; 2-tailed, paired Student's t-test). Values indicate means and SEM.

Figure 3. Overall effects of H₂O₂ on extracellular levels of 2'-AMP and correlation between extracellular 2'-AMP and adenosine in rat primary microglia.

To increase robustness and provide a better estimate of the effects of H₂O₂ on extracellular levels of 2'-AMP in rat primary microglia, we combined the results from **Study 1** and **Study 2**. As shown in panel A, the *P*-value for the effects of H₂O₂ on extracellular 2'-AMP reached *P*=0.000005 (2-tailed, paired Student's *t*-test). This robust result leaves little doubt that H₂O₂ causes a remarkable increase in extracellular levels of 2'-AMP. Also shown is a linear regression analysis between extracellular levels of 2'-AMP and extracellular levels of adenosine (panel B). Notably, 2'-AMP explained 52% of the variation in extracellular adenosine levels (*P*<0.0001). Results were normalized to cell number, and values indicate means and SEM.

Figure 4. H₂O₂ induced oxidative stress in microglia.

To confirm that 40 μ M of H₂O₂ induced oxidative stress in microglia we employed the OxyBlot™ Kit. This method uses the reagent 2,4-dinitrophenylhydrazine (DPH) to derivatize carbonyl groups (introduced by oxygen free radicals) in protein side chains to 2,4-dinitrophenylhydrazone, which is detected by western blotting with a primary antibody to the 2,4-dinitrophenylhydrazone moiety. As shown, a positive control provided by the kit gave a clear signal, indicating that the assay system was sensitive. Equal amounts of protein from microglia treated or not with H₂O₂ were processed either in the presence of DPH or without DPH (negative control). As shown, in the presence of DPH, microglia pretreated with H₂O₂ gave a much darker image compared with microglia not treated with H₂O₂. Importantly, no signal was observed in protein samples processed in the absence of DPH (negative control). These experiments confirm that 40 μ M of H₂O₂ indeed induced oxidative stress.