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RESEARCH ARTICLE

Caffeine Induces Adenosine A2A Receptor Triggering Brain Cell Damage in A172 Brain Cells

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ABSTRACT: The Adenosine A2A Receptor, ADORA2A, is a protein-coding gene that plays a crucial role in neuromodulatory functions. High expression of the A2A receptor in human brain cells is one of the known stress biomarkers in the human brain. Furthermore, a high dose of caffeine can cause brain diseases like stroke and dementia. However, the effect of a high dose of caffeine on ADORA2A gene expression in human brain cells has never been investigated. Therefore, we hypothesized that caffeine might induce stress through an increased intracellular H_2O_2 level, which overexpresses ADORA2A in brain cells. First, when A172 human brain cells were incubated with various concentrations of caffeine, the caffeine-treated A172 cells showed a significantly higher ADORA2A expression level than the no caffeine-treated control sample. Second, when A172 human brain cells were treated with 0 μM caffeine and 20 μM caffeine, the 20 μM caffeine sample had a 3-fold increase in its H_2O_2 levels, demonstrating that caffeine increases intracellular H_2O_2 levels in A172 cells. Third, 30 μM and 50 μM of H_2O_2 treatment significantly increased the ADORA2A expression level, indicating that a higher H_2O_2 concentration leads to a higher ADORA2A expression level. Overall, we found that high doses of caffeine significantly augmented the ADORA2A expression levels through increased intracellular H_2O_2 in human brain cells. The application of this novel molecular mechanism may contribute to the development of therapeutic drugs for caffeine-induced stress in human brain cells..

KEYWORDS: Biology; Neuroscience; Caffeine; Brain Damage; ADORA2A receptor.

Introduction

ADORA2A encodes Adenosine A2A Receptor, which plays a crucial role in neuromodulatory functions and facilitates neurotransmissions. When experiencing an increased workload or a significant amount of stress, the body uses a greater amount of intracellular ATP, increasing firing rate conditions and thus selectively activating A2A receptors.[1] Furthermore, ATP also acts as a danger signal in the brain, prompting changes in neuronal circuits in which more extracellular ATP is produced.[2] The high concentration of synaptic extracellular adenosine selectively activates Adenosine A2A Receptor.[3] Then, it promotes synaptic plastic changes in neuronal circuits that contribute to neurotoxicity in the brain.

Pharmacological and genetic evidence demonstrates that when suffering from an acute brain injury or chronic brain disease, the overactivation of Adenosine A2A Receptors can trigger brain dysfunction.[4] Therefore, Adenosine A2A Receptor antagonists showed a neuroprotective effect in different brain damage models, including Alzheimer's disease, ischemic brain stroke, and Parkinson's disease.[5]

While many people nowadays drink coffee to boost their alertness and enhance brain function, caffeine can actually cause brain damage.[6] According to a study at UniSA's Australian Centre for Precision Health, there was a significant association between reduced brain volume and the amount of coffee consumed.[6] Furthermore, excessive drinking of coffee has been shown to put people at risk of various brain diseases, including stroke and dementia. In addition to these long-term damages, caffeine can also reduce cerebral blood flow and induce brain hypoperfusion, thus posing short-term and long-term risks to its consumers.[7]

On the contrary, other studies suggest that drinking caffeine can protect the brain from the risk of brain diseases. In one study, positron emission technology (PET) scans demonstrated that regular consumption of coffee led to adenosine receptors being blocked by caffeine.[8] If this blockage persists over time, it can lead to adaptations in the brain, which change the receptor's availability. This chronic shift can lead to a decreased risk of brain diseases like dementia.[8] Overall, caffeine can have both positive and negative effects on the brain depending on various factors, including the amount consumed. Thus, in this study, we aimed to focus on how high dosages of caffeine would impact the brain.

Previous research indicated that caffeine induces hydrogen peroxide in high dosages. Therefore, in this study, we hypothesized that caffeine would induce intracellular stress in human brain cells through an increased intracellular H_2O_2 level, which may increase ADORA2A expression. Since ADORA2A is a well-known brain cell stress maker, we focused on the ADORA2A expression level as an indicator of caffeine-induced cells in human brain cells.

Methods

Cell culture and maintenance:

A172 human brain cells were purchased from Korea Cell Line Bank. The cells were maintained in the RPMI1640 cell culture media (Welgene) supplemented with 5% pen strep and

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10% fetal bovine serum. The cells were kept healthy under a 37 ˚C cell incubator with 5% CO_2.

RNA extraction:

The total RNA was extracted from the A172 cells using AccuPrep® Universal RNA Extraction Kit (Bioneer), followed by the manufacturer's provided instructions. 50 µL eluent was used for the downstream experiments.

Agarose gel-based RT-PCR:

After cDNA was synthesized using the extracted RNA by the TOPscript™ Reverse Transcriptase kit (Enzynomics), a 20 µL PCR reaction was performed to amplify ADORA2A and GAPDH, which is used for normalizing ADORA2A expression level. The following PCR reaction was used to synthesize both genes. For annealing temperature, 62 ˚C was used, and the extension time was set to be 20 sec. A total of 30 cycles were run using a Thermocycler machine (Bioneer).

Intracellular H_2O_2 staining:

The intracellular H_2O_2 staining solution (Invitrogen) was used to stain H_2O_2 in green fluorescence. DAPI staining solution was used to stain the nucleus in blue fluorescence. After the treatment, each sample was incubated with a one-hour staining solution inside the 5% CO_2 cell incubator. ImageJ was used to quantify the H_2O_2 level. The "Threshold" tool was used to identify and select the fluorescence signal in the image. This tool sets a threshold intensity level and will highlight all pixels in the image that exceed this intensity. "Analyze Particles" was used to measure the size and intensity of the fluorescence signal. This tool generated a table of measurements, including the area, mean intensity, and integrated density of each fluorescence signal. Lastly, the "Measure" tool was used to manually measure the fluorescence intensity at specific locations in the image.

Statistical analysis :

One-way ANOVA with Tukey's post hoc test was used to calculate the p-value.

Results and Discussion

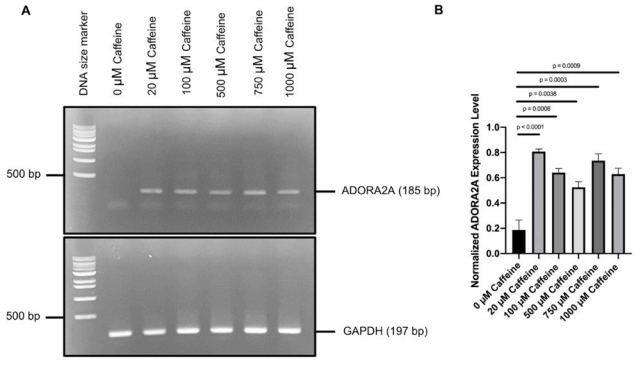

Figure 1: Caffeine increased ADORA2A mRNA expression level in A172 human brain cells. (A) Agarose gel image representing the amplified band ADORA2A and GAPDH cDNA. (B) Bar graph illustrating the mean and standard deviation of normalized ADORA2A mRNA expression level. GAPDH expression level was used to normalize ADORA2A expression level (n = 2). One-way ANOVA with Tukey's post hoc test was used to calculate the p-value.

Through this first experiment, we aimed to investigate the correlation between caffeine and ADORA2A mRNA expression levels in human brain cells. After incubating A172 brain cells for five days with cell culture media, we added caffeine solutions of different concentrations to five samples: 0, 20, 100, 500, 750, and 1000 µM. After performing RT-PCR on these solutions, we then amplified cDNA samples and loaded them in agarose gel, allowing us to analyze the respective band intensities. No discernible band appeared for the sample with 0 µM caffeine (Figure 1A). On the other hand, an evident band of amplified ADORA2A showed for the samples treated with caffeine, demonstrating that caffeine treatment increased the ADORA2A expression level. Furthermore, all caffeine-treated cells showed a significant increase in their ADORA2A expression level compared to the control sample with 0 µM caffeine (Figure 1B). Thus, the experiment results demonstrated that caffeine increases ADORA2A mRNA expression levels in A172 human brain cells.

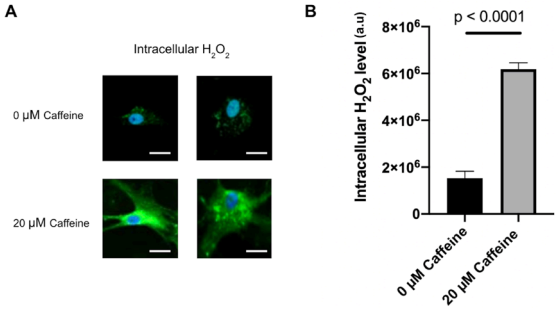

Figure 2: Caffeine increased the intracellular H_2O_2 in A172 human brain cells. (A) Fluorescence images indicating nucleus (blue) and intracellular H_2O_2 (green) in A172 cells (B) Bar graph representing mean and standard deviation of intracellular H_2O_2 level in A172 cells. (n = 2). One-way ANOVA with Tukey's post hoc test was used to calculate the p-value.

Through this experiment, we sought to test whether caffeine affects intracellular H_2O_2. We tested two conditions: 0 µM caffeine and 20 µM caffeine, incubating them for 48 hours with A172 brain cells. After the experiment, we checked the intracellular H_2O_2 levels through the green fluorescence representing them. The samples treated with 20 µM caffeine showed stronger fluorescent signals when compared to the 0 µM caffeine treated samples (Figure 2A). Moreover, quantifying the green fluorescence levels revealed that the H_2O_2 levels had a 3-fold increase in the 20 µM caffeine-treated sample (Figure 2B). This result indicates that caffeine increased intracellular H_2O_2 levels in A172 cells.

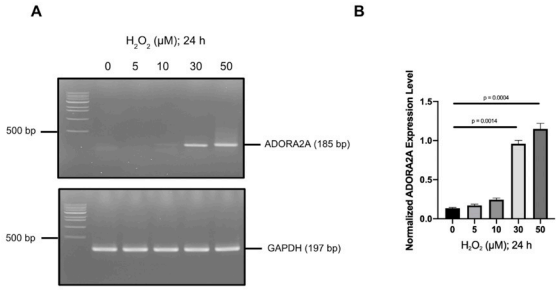

Figure 3: H_2O_2 increased ADORA2A mRNA expression level in A172 human brain cells. (A) Agarose gel image representing the amplified band ADORA2A and GAPDH cDNA. (B) Bar graph illustrating the mean and standard deviation of normalized ADORA2A mRNA expression level. GAPDH expression level was used to normalize ADORA2A expression level (n = 2). One-way ANOVA with Tukey's post hoc test was used to calculate the p-value.

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Based on the results of the previous experiments, which demonstrated a positive correlation of caffeine with H_2O_2 and ADORA2A, we aimed to investigate caffeine's effect on ADORA2A expression levels. Therefore, we hypothesized that caffeine might increase the ADORA2A expression level. After incubating A172 brain cells with 0, 5, 10, 30, and 50 μM H_2O_2, we used agarose gel and its band intensities to quantify the level of ADORA2A in each sample. While the A172 brain cells with H_2O_2 0, 5, and 10 μM did not significantly affect the ADORA2A band intensity, the cells incubated with H_2O_2 concentrations of 30 and 50 μM significantly increased the band intensity of ADORA2A (Figure 3A). Furthermore, the cells with H_2O_2 concentrations of 50 μM had a normalized ADORA2A expression level approximately nine times greater than that of the 0 μM H_2O_2 cells, demonstrating the positive correlation between H_2O_2 concentration and ADORA2A expression level (Figure 3B). Thus, the results of this experiment indicate that a higher level of intracellular H_2O_2 increases ADORA2A expression levels in A172 human brain cells.

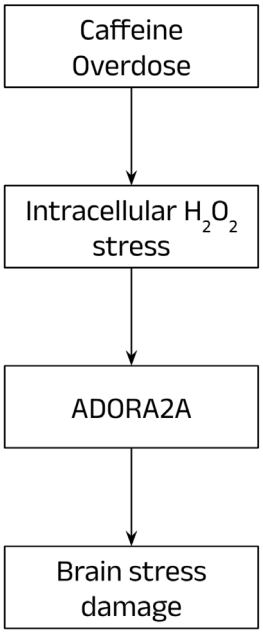

Figure 4: Discovery of a novel pathway for how caffeine overdose damages the human brain. When caffeine overdose induces intracellular H_2O_2 stress, ADORA2A expression is increased, and finally, brain stress damage is induced.

Based on the first experiment, caffeine caused an increase in ADORA2A mRNA expression levels in A172 human brain cells. Furthermore, caffeine also increased the intracellular H_2O_2 levels in the cells. Results from a subsequent experiment indicated that higher levels of intracellular H_2O_2 levels increase ADORA2A expression levels in A172 human brain cells. Overall, this study demonstrated a positive correlation between caffeine, intracellular H_2O_2 levels, and ADORA2A expression levels in human brain cells (Figure 4).

Conclusion

Though our study demonstrates the effects of caffeine on A172 human brain cells, we must validate this by testing different human brain cells and ensuring that our conclusion is not uniquely applicable to A172 brain cells. Conducting additional tests to uncover the detailed molecular mechanism of caffeine inducing such changes will also allow us to further our finding that caffeine increases intracellular H_2O_2 levels. Additionally, based on our finding that H_2O_2 levels increased the ADORA2A expression level, we expect that future studies will allow us to find the H_2O_2-dependent transcription factors that may have directly induced the change in ADORA2A expression levels.

With nine in ten Americans reporting consumption of caffeine and three in four having caffeine at least once a day, it is evident that many Americans consume caffeine regularly.[9] However, one in four consumes it three or more times a day, perhaps going beyond healthy consumption and raising concerns about caffeine safety and overdose.[10] Despite our familiarity with caffeine, there is not enough awareness of these concerns, and studies on the effects of caffeine on brain cells are minimal. Our study found that a caffeine overdose can induce ADORA2A, a brain stress marker, indicating that a caffeine overdose may damage the human brain. In addition to this finding, we also discovered details on how caffeine may damage the brain cells; caffeine increases ADORA2A expression through increased intracellular H_2O_2 levels. Since this is the first study of discovering a concrete, detailed mechanism regarding how caffeine can damage the brain, it expands opportunities for further study of caffeine in the future. Our discovery of this mechanism involving H_2O_2 will facilitate efforts to develop novel therapeutic drugs, allowing not only the awareness of how caffeine overdose tangibly impacts our brain but also enabling the minimization of future risks.

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