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Studies on the Mechanism of Caffeine Action in Alveolar Macrophages: Caffeine Elevates Cyclic Adenosine Monophosphate Level and Prostaglandin Synthesis

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We have previously reported that the effects of caffeine on alveolar macrophages are dose-dependent; thus, at low concentrations caffeine prevents apoptosis and at moderate concentrations, the cells proceed into apoptosis. In the current study, the mechanism of caffeine action via prostaglandin synthesis and cyclic adenosine monophosphate (cAMP) was investigated using moderate concentrations of caffeine. The results show that the combination of caffeine with indomethacin, an inhibitor of prostaglandin synthesis, mediated caffeine's effect by increasing cellular viability and lowering superoxide anion production and DNA fragmentation. However, addition of exogenous prostaglandin E2 (PGE2) to the culture in the presence of caffeine had the opposite effect, in which the viability was decreased and anion superoxide production was increased. Incubation of macrophages with exogenous dibutyryl cAMP showed nearly similar effects to caffeine. At low concentrations (<50 μmol/L), higher viability and lower superoxide production pattern were evident and at higher concentrations (>50 μmol/L) the cells proceeded into apoptosis. Therefore, it is suggested that caffeine exerts its effects on macrophages by altering cAMP level and prostaglandin synthesis.

CAFFEINE (1,3,7 trimethylxanthine) is the most widely consumed, behaviorally active substance in the world. The main sources are coffee, tea, and caffeinated cola drinks.[1-3] The diuretic, respiratory, cardiovascular, and central stimulant properties of caffeine have been known for decades. Caffeine has been used therapeutically to treat narcolepsy, asthma, and apnea, and as an analgesic adjunct.[4-6] The effects of caffeine in both humans and animals are biphasic in nature. In humans, low doses elicit a central stimulation and high doses elicit unpleasant effects, sometimes referred to as "caffinism."[2,7] The main mechanism of action of caffeine occurring at circulating concentration is the antagonism at the level of adenosine receptors, while higher doses would be required for significant inhibition of phosphodiesterases, the enzyme that breaks down cyclic adenosine monophosphate (cAMP).[8-10] Also, caffeine can stimulate prostaglandin synthesis, which induces an increase of cAMP level.[11,12]

Alveolar macrophages are the most abundant phagocytic cells in the lung, and have adopted important functions in the induction and regulation of acquired (humoral and cellular) immune reaction and in the control of cell functions of many nonphagocytic cells.[13,14] Our previous results have shown that the effect of caffeine on alveolar macrophages is dose- and time-dependent. At low concentrations, caffeine prevents apoptosis, whereas at moderate concentrations it induces apoptosis.[15] However, the mechanism of this effect of caffeine is unknown.

In the present study we planned to investigate the mechanism of caffeine action on cell survival, superoxide anion production, and DNA fragmentation in alveolar macrophages using exogenous indomethacin, prostaglandin E2 (PGE2), and cAMP. We conclude that caffeine exerts its function via cAMP and prostaglandin synthesis.

MATERIALS AND METHODS

Chemicals

Trypan blue, cytochrome C, superoxide dismutase (SOD), dibutyryl cAMP, and PGE2 were purchased from Sigma Chemical Co (St Louis,

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Mo). Diphenylamine (DPA) and other chemical compounds were obtained from Merck. Dulbecco's modified eagle medium (DMEM) and fetal calf serum (FCS) were from Gibco. DMEM supplemented with 30 mg/L of asparagine and antibiotics (120 mg/mL penicillin and 200 mg/mL streptomycin) pH 7.4 was prepared, and after sterilization by 0.45- μm millipore filters kept at 4°C until use. Caffeine (Merck) was freshly dissolved in DMEM culture medium immediately before use. PGE2 and indomethacin were dissolved in absolute ethanol at a stock concentration of 1 mg/mL and 0.5 mg/mL, respectively; appropriate dilutes were made using DMEM before use.

Animals

Male Sprague-Dawley rats (Hesarak Institute, Tehran, Iran) weighting of 200 to 250 g were used throughout the experiments.

Alveolar Macrophage Collection

Alveolar macrophages were collected from rat lungs using the bronchoalveolar lavage procedure previously described.[16] Lungs were lavaged 5 to 6 times each with 10 mL saline. The cells were collected by centrifugation at 2,000 $\times$ *g* for 10 minutes (4°C), washed once with DMEM medium supplemented with 10% FCS, and examined under microscope. Methylen green was used for cell count and cell viability was determined by trypan blue exclusion.

Treatment of Cells With cAMP, PGE2, and Indomethacin Combined With Caffeine

Freshly isolated cells were suspended at a density of 2 $\times$ 10^6 cells/mL in DMEM with 10% FCS and 1 mL suspension in duplicate and preincubated for 30 to 45 minutes in plastic Petri dishes (Nunc) at 37°C in a 95% air, 5% CO_2 in atmosphere and fully humidified condition. Caffeine, cAMP, indomethacin, PGE2 or a combination of them at desired concentrations were then added to the cultures, which were mixed thoroughly and incubated for 24 hours under the condition noted above.

Assay for Superoxide Release

After incubation of macrophages in the absence and presence of various concentrations of compounds for 24 hours, the cells were collected by rubber policeman followed by centrifugation for 10 minutes at 2,000 $\times$ *g*. The packed cells were washed twice with phosphate-buffered saline (PBS) and then to each sample 200 μL cytochrome C (160 μmol/L) and 200 μL phorbol 12-myristate 13-acetate (10^{-6} mol/L) were added. Also to the control was added 17 μL (1 μg/mL) SOD (60 U). The samples were incubated at 37°C for 15 minutes and then centrifuged at 6,000 $\times$ *g* for 10 minutes at 4°C. Superoxide anion production was determined from absorbance reading against the control at 550 nm using U.V 260 Shimadzu spectrophotometer.[17]

Quantitative Analysis of DNA Fragmentation

Treated alveolar macrophages and controls were collected by centrifugation at 2,000 $\times$ *g* for 10 minutes at 4°C. The cell pellets were lysed in 0.5 mL lysis buffer containing 10 mmol/L tris-HCI (pH 8), 1 mmol/L EDTA, and 0.2% Triton X-100, and centrifuged at 10,000 rpm (Eppendorf) for 20 minutes at 4°C. After resuspending in 0.5 mL of lysis buffer, to the pellets and the supernatants 0.5 mL of 25% trichloroacetic acid (TCA) was added and incubated at 4°C for 24 hours. The samples were centrifuged for 20 minutes at 10,000 rpm (Eppendorf) at 4°C and the pellets were suspended in 80 μL of 5% TCA, followed by incubation at 83°C for 20 minutes. Then to each sample 160 μL of DPA solution (150 mg DPA in 10 mL glacial acetic acid with 150 μL of sulfuric acid and 50 μL acetaldehyde 16 mg/mL) was added and incubated at room temperature for 24 hours.[18] The proportion of fragmented DNA was calculated from absorbance reading at 600 nm using the formula:

$$\% \text{ Fragmented DNA} = \text{OD (S)}/\text{OD (S)} + \text{OD (P)} \times 100.$$

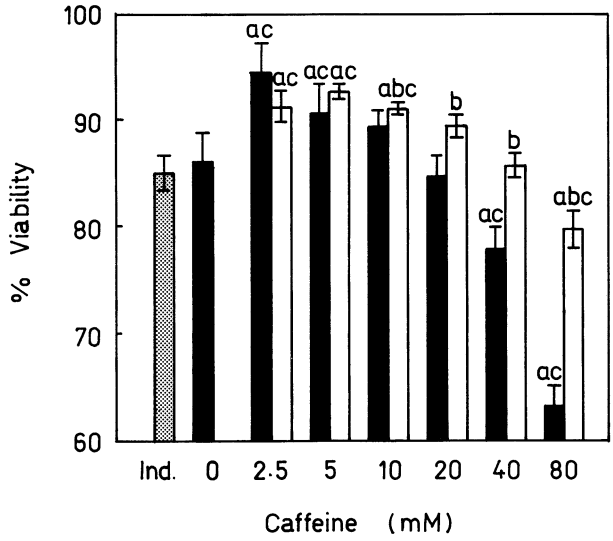

Fig 1. Effect of indomethacin in the absence and presence of various concentrations of caffeine on 2 $\times$ 10^6 alveolar macrophages from rat lung for 24 hours of incubation. (□) With indomethacin; (■) without indomethacin; Ind, indomethacin. Data are means ± SD of 4 experiments. [a]*P* < .05 *v* control (no caffeine); [b]*P* < .05 *v* the same concentrations of caffeine; [c]*P* < .05 *v* indomethacin (3 μmol/L).

Statistical Analysis

All calculations were performed using Instat statistical software. Results were analyzed by analysis of variance (ANOVA), paired *t* test, and unpaired *t* test. *P* values less than .05 were considered statistically significant. Results were expressed as means ± SD mean, with n denoting the number of experiments performed.

RESULTS

Macrophages obtained from rat lungs were 95% to 98% viable when examined under microscope and trypan blue exclusion procedure. Figure 1 shows the survival curve of the cells cultured for 24 hours with various concentrations of caffeine in the absence and presence of 3 μmol/L indomethacin, as an inhibitor of prostaglandin synthesis. At low concentrations of caffeine (<5 mmol/L) alone, viability was markedly enhanced and the peak of enhancement was observed at 2.5 mmol/L. Between 5 mmol/L and 15 mmol/L of caffeine, the viability of the cells was still higher than the control level. At 20 mmol/L it reached to the control level and at higher concentrations caffeine inhibited cell survival and killed a fraction of the population. The viability pattern of low concentrations of caffeine (<5 mmol/L) combined with indomethacin (3 μmol/L) was nearly similar to caffeine-alone samples, but at higher concentrations of caffeine (≥10 mmol/L) viability was increased and the maximal effect was observed at 80 mmol/L, which represented 79.8% of the viability (compared to 80 mmol/L, caffeine-treated cells of 63%, $P < .001$). The viability

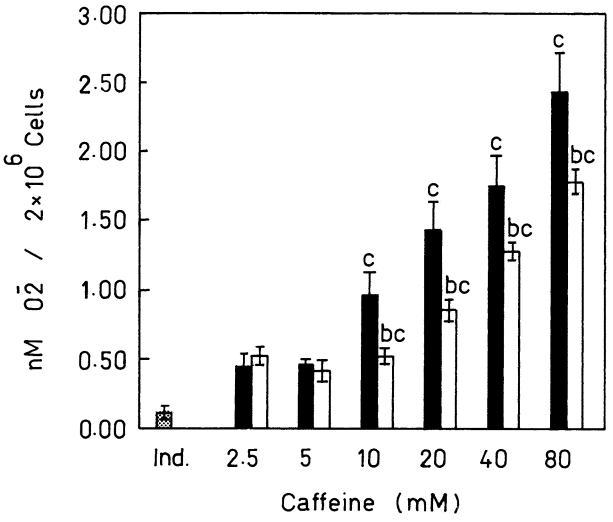

Fig 2. Effect of indomethacin in the absence and presence of various concentrations of caffeine on superoxide anion release from alveolar macrophages incubated for 24 hours. (□) With indomethacin; (■) without indomethacin; Ind, indomethacin. Data are means ± SD of 4 experiments. $^bP < .05$ *v* the same concentrations of caffeine; $^cP < .05$ *v* indomethacin (3 μmol/L).

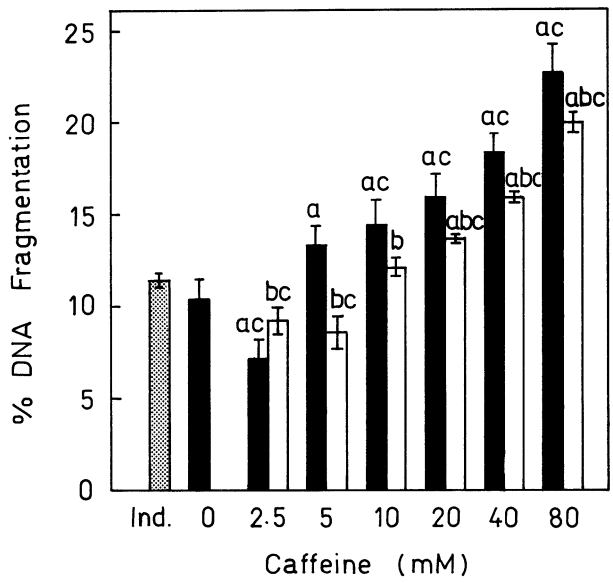

Fig 3. Effect of indomethacin in the absence and presence of various concentrations of caffeine on DNA fragmentation from alveolar macrophages incubated for 24 hours. (□) With indomethacin; (■) without indomethacin; Ind, indomethacin. Data are means ± SD of 4 experiments. $^aP < .05$ *v* control (no caffeine); $^bP < .05$ *v* the same concentrations of caffeine; $^cP < .05$ *v* indomethacin (3 μmol/L).

of the cells in the presence of indomethacin alone (3 μmol/L) showed no significant change compared to the control.

Determination of superoxide anion production of macrophages cultured in the absence and presence of caffeine and in combination with indomethacin showed that up to 2.5 mmol/L caffeine alone, no significant SOD-inhibitable reduction of cytochrome C was observed. At higher concentrations of caffeine (>5 mmol/L), the superoxide production was augmented, but in combination with indomethacin (3 μmol/L), the rate of superoxide anion production decreased; thus, at 80 mmol/L, 1.782 nmol anion production was obtained (Fig 2). In the presence of indomethacin alone (3 μmol/L) there was no significant change in the rate of O_2^- production.

Quantitative DNA fragmentation analysis determined by DPA reaction to the procedure is presented in Fig 3. Cells exposed to high concentrations of caffeine (≥10 mmol/L) showed a profound increase in DNA fragmentation. As at 80 mmol/L, nearly 2-fold increase was observed when compared to the control with no caffeine. However, caffeine in combination with indomethacin showed a significant decrease in DNA fragmentation ($P < .05$). In the presence of indomethacin alone, the rate of DNA fragmentation was similar to the control.

To determine whether the effects seen above were the result of the inhibition of PGE2 synthesis, the effects of PGE2 on survival rate and superoxide anion production of macrophages cultured in the absence and presence of caffeine were examined. For simplicity, 2 concentrations of caffeine, 5 and 10 mmol/L, were chosen. Figure 4 shows the viability of caffeine-treated macrophages incubated for 24 hours in the absence and presence of various concentrations of PGE2. Addition of exogenous PGE2 (up to 10^{-7} mol/L) to the cultures increased the survival rate of macrophages ($P < .01$), but at 10^{-5} mol/L,

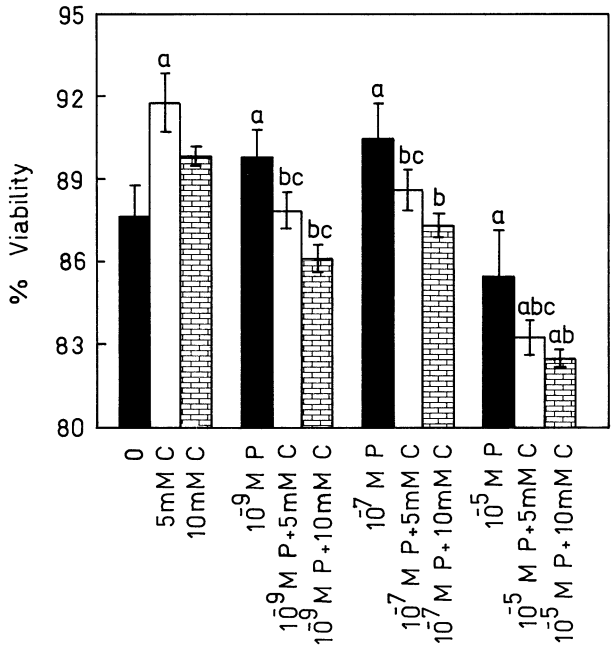

Fig 4. Effect of various concentrations of PGE2, 5 and 10 mmol/L caffeine on 2×10^6 alveolar macrophages from rat lung cultured for 24 hours. C, caffeine, P, PGE2. Results are expressed as the means ± SD of data from at least 4 separate experiments. $^aP < .05$ *v* control (no caffeine and no PGE2); $^bP < .05$ *v* the same concentrations of PGE2; $^cP < .05$ *v* the same concentrations of caffeine.

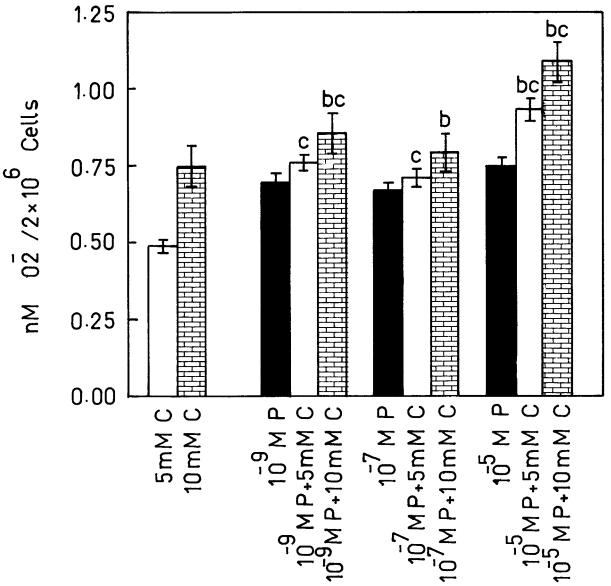

Fig 5. Effect of combination of PGE2 with 5 and 10 mmol/L caffeine on caffeine on superoxide anion release from alveolar macrophages at 2 × 10^6 cells/mL for 24 hours. C, caffeine; P, PGE2. Data represent means ± SD means of 3 experiments. $^bP < .05$ *v* the same concentrations of PGE2; $^cP < .05$ *v* the same concentrations of caffeine.

viability was markedly decreased ($P < .05$) when compared to the control with no PGE2. In the presence of both 5 and 10 mmol/L caffeine, viability of the cells was decreased, and at 10 mmol/L concentration, the effect was no longer visible.

Figure 5 shows the effect of PGE2 on superoxide anion release from alveolar macrophages cultured for 24 hours in the absence and presence of caffeine. Different concentrations of PGE2 showed different effects on superoxide anion production; thus, at 10^{-7} mol/L, O_2^- production was decreased and at 10^{-5} mol/L, significant increase was observed. However, in the presence of caffeine (5 and 10 mmol/L) and the 3 concentrations of PGE2, the rate of O_2^- production was increased considerably. The O_2^- production at 10 mmol/L caffeine and 10^{-5} mol/L PGE2 was higher than 5 mmol/L caffeine in the same condition. PGE2 at 10^{-7} mol/L concentration showed the lowest superoxide anion production level, which is in agreement with the results obtained for the viability test.

In has been shown that PGE2 induces an increase of cAMP level through activating adenylate cyclase.[11,12] In addition, caffeine is known as an inhibitor of phosphodiesterase.[3,10] Therefore, to study the effect of cAMP on alveolar macrophages and whether the effects observed above are exerted through changes in the cAMP level, macrophages were cultured in the presence of cAMP added exogenously to the media. Figure 6A illustrates the viability of cells incubated for 24 hours in the absence and presence of various concentrations of cAMP. In this case, a similar pattern to the effect of caffeine was obtained.[10] Low concentrations of cAMP (<50 μmol/L) enhanced viability, and at 50 μmol/L, viability was still higher than the control level. Higher concentrations of cAMP (≥100 μmol/L) markedly decreased the cell survival rate; thus, at 200 μmol/L, only 78% of the viability was retained ($P < .001$). In Fig 6B, the effect of cAMP on superoxide anion release and the percent of DNA fragmentation of alveolar macrophages is shown. Up to 30 μmol/L of cAMP, the superoxide anion production level was low and DNA fragmentation was decreased, but at higher concentrations (>50 μmol/L) both O_2^- production and DNA fragmentation were increased.

DISCUSSION

Macrophages are believed to play a major role in the development of the inflammatory process[19,20] and are known to release considerable amounts of prostaglandins.[21-23] Caffeine is an inhibitor of cyclic nucleotide phosphodiesterase, and in some tissues stimulates prostaglandin synthesis,[11,24] both of which lead to increased cAMP level.[22,23] Our previous results have shown that the effect of caffeine on cell survival, superoxide production, and DNA fragmentation of alveolar macrophages is dose- and time-dependent.[15] In the present study, the mechanism of caffeine action on macrophages via cAMP and prostaglandin biosynthesis was examined.

The results demonstrate that in the presence of caffeine alone, the cell survival rate at 2.5 mmol/L of caffeine was

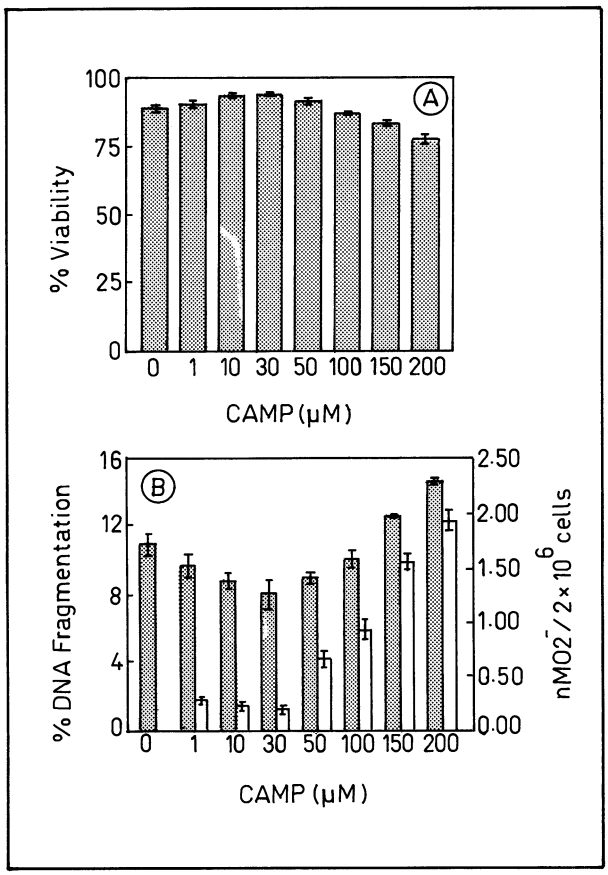

Fig 6. Effect of cAMP on (A) viability and (B) superoxide anion release and DNA fragmentation of alveolar macrophages cultured for 24 hours in DMEM in the presence of 10% FCS. (▨) Superoxide anion release; (□) DNA fragmentation. Results are means ± SD. (n = 3), $P < .05$.

increased remarkably, but at higher concentrations (>5 mmol/L) caffeine inhibited cell survival. When a combination of indomethacin with low concentrations of caffeine (≤5 mmol/L) was added to the cultures, there was no significant difference in the viability of the cells compared to the caffeine-treated cells, while higher concentrations of caffeine (≥10 mmol/L) increased the viability. Also, a significant decrease in both superoxide anion production and DNA fragmentation were observed. These results show that indomethacin diminishes the effect of caffeine. This is in agreement with the results reported by De Pasquale et al that indomethacin, as an inhibitor of prostaglandin biosynthesis, inhibits the effect of caffeine in rats.[25] Other studies have also shown that indomethacin induces apoptosis in different cells.[26,27]

The effect of PGE2 on the survival rate of alveolar macrophages is dose-dependent. The survival curves of cells in the presence of 10^{-9} and 10^{-7} mol/L PGE2 remained higher than the control, while at 10^{-5} mol/L, viability was decreased. When caffeine was added in combination with various PGE2 concentrations, cell viability was decreased. These results suggest that probably both PGE2 (by activating adenylate cyclase enzyme) and caffeine (by inhibition of phosphodiesterase) increase cAMP levels in alveolar macrophages.[12,28,29] On the other hand, it has been shown that an increase in cAMP level proceeds apoptosis.[30,31] This is in agreement with the results reported previously that caffeine inhibits phosphodiesterase enzyme following enhanced formation of PGE2,[32] and that PGE2 can cause apoptosis in various types of cells.[33-35] In other words, an increase in PGE2 synthesis elevates cAMP level.

Our results also show that the effect of cAMP on macrophages is dose-dependent. Low concentrations of cAMP (<50 μmol/L) enhanced cell survival; however, in the presence of higher concentrations of cAMP (>50 μmol/L), a significant decrease in cell survival was observed. Analysis of anion superoxide production and DNA fragmentation patterns indicated no significant increase using low concentrations of cAMP (<50 μmol/L), but at higher concentrations (>50 μmol/L) both superoxide anion production and DNA fragmentation were increased. The behavior of cAMP on metabolic activity of alveolar macrophages is very similar to the effects of caffeine on these cells, as reported previously.[15] Previous results reported on other cell types indicate that caffeine is relatively nontoxic and even at millimolar concentrations (2 mmol/L) does not immediately affect cell viability,[36,37] while at higher concentrations (>10 mmol/L) it produces chromosome breaks and kills the cells.[38,39] This result suggests that cAMP itself, at low concentrations, inhibits apoptosis[30,40] and at higher concentrations proceeds apoptosis.[30,31]

In conclusion, low concentrations of caffeine elevate cAMP levels in caffeine-treated macrophages, and possibly through this mechanism prevent apoptosis. On the other hand, higher concentrations of caffeine markedly elevate cAMP level and lead macrophages into apoptosis. Therefore, the results suggest that caffeine probably exerts its action through cAMP and prostaglandin synthesis in alveolar macrophages. However, the direct determination of cAMP and PGE2 levels in caffeine-treated cells remains to be established. Whether other mechanisms such as direct binding of caffeine to cyclooxygenase or arachidonic acid is not clear and demands further investigation to elucidate the real action of caffeine in alveolar macrophages.

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