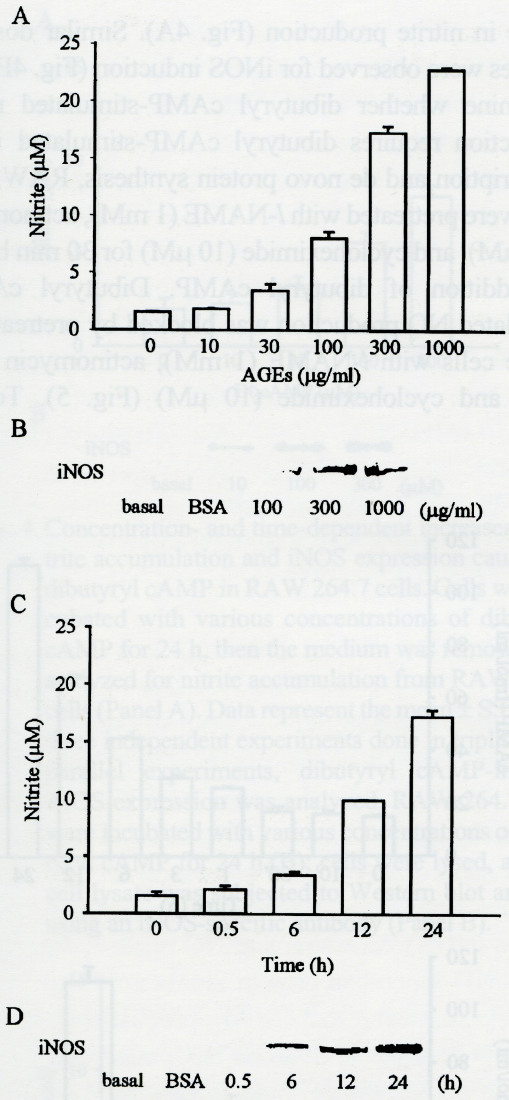

Fig. 1. Concentration- and time-dependent increases in nitrite accumulation and iNOS expression caused by BSA-AGEs in RAW 264.7 cells. Cells were incubated with various concentrations of BSA-AGEs for 24 h (Panel A), or with BSA-AGEs (300 μg/mL) for various time intervals (Panel C), then the medium was removed and analyzed for nitrite accumulation from RAW 264.7 cells. Data represent the mean ± S.E.M. of three independent experiments done in triplicate. In parallel experiments, AGE-induced iNOS expression was analyzed. RAW 264.7 cells were incubated with various concentrations of BSA-AGE for 24 h (Panel B), or with BSA-AGEs (300 μg/mL) for various time intervals (Panel D); cells were lysed, and the cell lysate was subjected to Western blot analysis using an iNOS-specific antibody.

dose-dependent manner (Fig. 1B). When cells were treated with a submaximal concentration (300 mg/mL) of BSA-AGEs for various time intervals, nitrite levels increased in a time-dependent manner, concomitant with upregulation of the iNOS protein, which began at 6 h and reached a maximum at 24 h (Fig. 1C, D). In the following experiments, cells were treated with 300 μg/mL of BSA-AGEs for 24 h.

Involvement of the PKA signaling pathway in AGE-stimulated iNOS expression and nitrite production by RAW264.7 cells

To investigate whether cAMP-dependent protein kinase (PKA) is involved in the AGE-stimulated induction of iNOS, PKA inhibitors (KT 5720 or H-8) were used to pretreat cells. When cells were pretreated with KT 5720 for 30 min, AGE-induced nitrite release and iNOS production were inhibited by KT 5720 (0.03-1 μM) in a dose-dependent manner (Fig. 2A). Similar results were observed when cells were pretreated with another PKA inhibitor, H-8 (0.3-10 μM) (Fig. 2B). Consistently, AGE-induced iNOS expression was attenuated by pretreatment with H-8 (3 or 10 μM) or KT 5720 (0.3 or 1 μM) (Fig. 2C). These results suggest that cAMP-dependent protein kinase is involved in AGE-stimulated NO release.

AGE-stimulated cAMP formation in RAW 264.7 macrophages

Because the PKA pathway seems to be involved in AGE-induced NO production and iNOS induction, intracellular cAMP was assayed following BSA-AGEs (300 μg/mL) treatment for various time periods (Fig. 3A). cAMP formation was apparent at 1 h, with a maximum at 24 h after the BSA-AGEs were added, and gradually decreased thereafter (data not shown). When cells were treated for 24 h with various concentrations of AGEs, AGEs stimulated cAMP accumulation in a dose-dependent manner (Fig. 3B).

Effects of dibutyryl cAMP on NO production and iNOS induction in RAW 264.7 cells

Because cAMP-dependent protein kinase seems to be involved in AGE-stimulated NO release, dibutyryl cAMP was used to activate PKA, and its effects on nitrite