least 6 months. All incubations were performed under sterile conditions in the dark at 37 °C. After incubation, unreacted sugars were removed before assaying by extensive dialysis against PBS. The BSA-AGEs solution was filter-sterilized and stored in a freezer before use.

Culture of RAW 264.7 cells and preparation of cell lysates

RAW 264.7 cells of a murine macrophage cell line, were cultured in Dulbecco Modified Eagle Medium/F-12 (D-MEM/F-12) supplemented with 2.438 g/L $NaHCO_3$, 2 mM L-glutamine, 1 mM sodium pyruvate, 10% FBS, and penicillin (100 U/mL)/streptomycin (100 mg/mL). Cultures were maintained in a humidified incubator with 5% CO_2 at 37 °C. Cells were plated at a concentration of 1×10^5 cells/mL and used for the experiment when they reached 80% confluency. Cells were harvested, chilled on ice, and washed 3 times with ice-cold PBS. Cells were lysed in lysis buffer containing 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 100 μg/mL phenylmethylsulfonyl fluoride (PMSF), 1% Nonidet P-40 (NP-40), and 4% protease inhibitor cocktails. Protein concentrations in the cell lysates were determined using a Bio-Rad protein assay following the manufacturer's instructions. All cell lysates were stored at -70 °C until further measurements.

Polyacrylamide gel electrophoresis and Western blotting

Electrophoresis was ordinarily carried out on different percentages of SDS-polyacrylamide electrophoresis gels (SDS-PAGE). Following electrophoresis, separated proteins on the gel were electrotransferred onto a polyvinyldifluoride (PVDF) membrane. Nonspecific IgGs were blocked with blocking buffer containing 5% fat-free milk powder in TBST for 1 h at room temperature, followed by incubation with primary antibodies in blocking buffer for 2 h. The PVDF membrane was then incubated with alkaline phosphatase-conjugated secondary antibodies for 1 h. Subsequently, the Western blots were developed with 5-bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium (BCIP/NBT) as the substrate.

Measurement of nitrite formation in RAW 264.7 cell cultures

Confluent RAW 264.7 cell cultures in 35-mm Petri dishes were incubated in fresh medium for 24 h under the experimental conditions indicated in Results. Nitrite production was measured by addition of 0.15 mL of the cell culture medium to 0.15 mL of Griess reagent (Gross et al., 1991) in a 96-well plate, and incubation in a dark place at 37 °C for 10 min. Absorbance was measured at 540 nm using a microplate reader. A blank was prepared for each experimental condition in the absence of RAW 264.7 cells, and the absorbance was subtracted from that obtained in the presence of cells.

Measurement of intracellular cAMP in RAW 264.7 cell cultures

RAW 264.7 cells were pretreated with inhibitors for 30 min before incubation with 300 μg/mL BSA-AGEs for indicated time periods. The reaction was terminated by removing the medium and adding 0.1 N HCl. Cells were scraped, and the suspension was centrifuged; the supernatants were assayed for cAMP level using an enzyme immunoassay kit after neutralization with NaOH (10 N) (Amersham Pharmacia Biotech).

Statistics

All data are expressed as the mean ± S.E.M. Comparisons between groups were made by Student's *t*-test. A difference between groups of $p < 0.05$ was considered significant.

RESULTS

AGEs stimulated dose- and time-dependent increases in NO release and iNOS expression

Exposure of RAW264.7 macrophages to AGEs stimulated nitrite production in a dose-dependent manner (Fig. 1A). To determine whether the elevated nitrite production was related to upregulation of iNOS expression, Western blotting analysis was performed using iNOS-specific antibodies. AGEs induced the expression of 130-kDa iNOS in RAW 264.7 cells in a