TUNEL assay. Apoptotic cells in the BRECs cultures were detected using the TUNEL System (Promega, Madison, WI) according to the manufacturer's instructions. The TUNEL-positive cells were then photographed under a Zeiss Axioplan 2 fluorescence microscope with a digital Zeiss AxioCam color camera (Carl Zeiss, Göttingen, Germany), and their counts were estimated in 5 fields (1 in the center and 1 in each of the 4 corners) in each of the 3 dishes for each treatment condition. Each condition was assessed at least in triplicate, and the experiments were independently repeated 3 times.

Isolation of the mitochondrial fraction from rat retina

Mitochondria were isolated from rat retina as described by our previous study (Diabetes 58:954-964, 2009). Retinas obtained from eight rat eyes were washed in buffer A containing 225 mM mannitol, 75 mM sucrose, l mM EGTA, and 5 mM HEPES (pH 7.0). Retinas were placed in a glass Teflon homogenizer containing 15 mL buffer A and were homogenized (six strokes). Buffer A (20 mL) containing 2 mM EGTA and 1% BSA was added and centrifuged at 1000g for 5 minutes. The supernatant was diluted 1:1 with buffer A and centrifuged again at 1000g for 5 minutes. The pellet was discarded, and the supernatant was centrifuged at 11,500g for 15 minutes. The pellet was resuspended in buffer A (without EGTA) and centrifuged again at 11,500g for 15 minutes. Finally, the pellet containing the mitochondria was resuspended in buffer A (without EGTA) and either used on the same day or frozen in liquid nitrogen for storage at -70°C. The freshly isolated mitochondria were approximately 80% intact, as indicated by their succinate-cytochrome c oxidoreductase activity before and after hypotonic shock. This mitochondrial preparation is well coupled, with a respiration control ratio of 2.44 ± 0.2 ($n = 3$), as determined from the rate of succinate-supported electron transport in the absence and presence of gramicidin.

Isolation of the mitochondrial fraction from cultured cells

Cells are washed in PBS and harvested in log-phase by scraping. The cells are precipitated by an LSS (500g for 12 min) and 1 wet volume of MSHE buffer is then added. Using a dounce glass–glass homogenizer instead of a standard glass–teflon homogenizer appears to increase the yield from cultured cells. Since mechanical homogenization of cells is less efficient than tissue, 0.01% digitonin can be added to the MSHE buffer using a modified version of Greenawalt's method (Methods Enzymol 31: 310-323, 1974) as follows: to check the homogenization efficiency a 10 μL aliquot of the homogenate is added to 20 μL trypan blue and inspected under microscope. At least 90% of the cells should take up the dye. If this is not the case another 0.01% of digitonin can be added and further homogenization can be performed. Several steps are sometimes necessary for complete homogenization. To ensure purity of the mitochondrial fraction it can occasionally be necessary to add a protease to digest cytosolic and nuclear proteins before the mitochondria are lysed. We have used subtilisin (1.4 U/mL) during the homogenization for this purpose.

Measurement of ROS. ROS production in the cells was assessed using the fluorescent probe

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5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA; Molecular Probes, Eugene, OR). CM-H_2DCFDA (λ_{ex}, 488 nm and λ_{em}, 520 nm) is a cell-permeable indicator for ROS that remains non-fluorescent until both the acetate groups have been removed by intracellular esterases and oxidation has occurred within the cell. The cell suspension (200 µl at ~10^5 cells/ml) was incubated in a FluoroNunc 96-well polystyrene plate with CM-H_2DCFDA (10 µM) for 45 min at 37°C. Intracellular ROS production was calculated using an H_2O_2 standard curve (10–200 nmol/ml). Retinal mitochondria ROS production was detected as described by our previous study (Diabetes 58:954-964, 2009). Retinal tissues were harvested in cold-buffered medium (5 mmol/l HEPES in PBS) and immediately frozen in liquid nitrogen to improve the following probe diffusion. After rapid thawing, medium was discarded. Samples were exposed to 8 µM CM-H2DCFDA dissolved in 400 µl fresh medium and were incubated at 37°C for 30 min under agitation. Medium was then removed, and samples were further incubated in a lysis buffer (0.1% SDS, Tris-HCl, pH 7.4) for 15 min at 4°C. After homogenization, samples were centrifuged at 6,000g for 20 min at 4°C. Supernatants were collected and subjected to fluorescence analysis as stated above.

Fig. 2G. Immunohistochemical staining for p-p38MAPK in trypsin-digested retinal blood vessels in control rats (C), diabetic rats (DM) and diabetic rats treated with simvastatin (DM + S). Sections were counterstained with hematoxylin. Original magnification was 400×. Bars indicate SD. A representative experiment of the three is shown ($^{**}P < 0.01$ vs. control, $^{\#\#}P < 0.01$ vs. DM, n=8).

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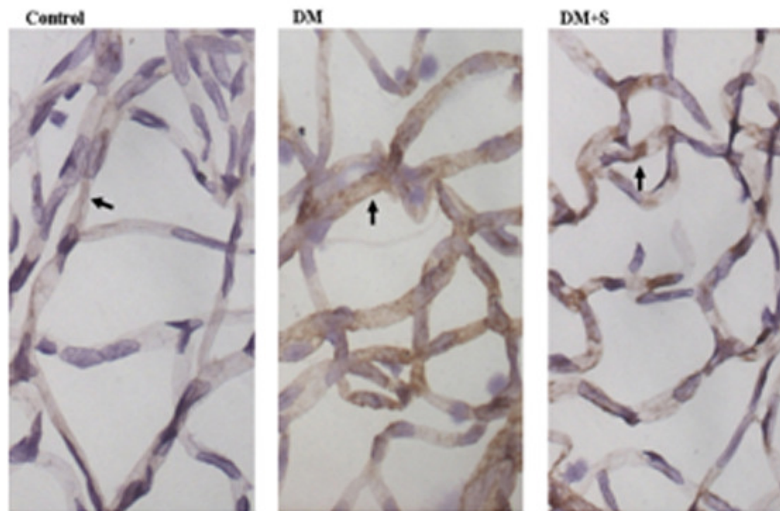
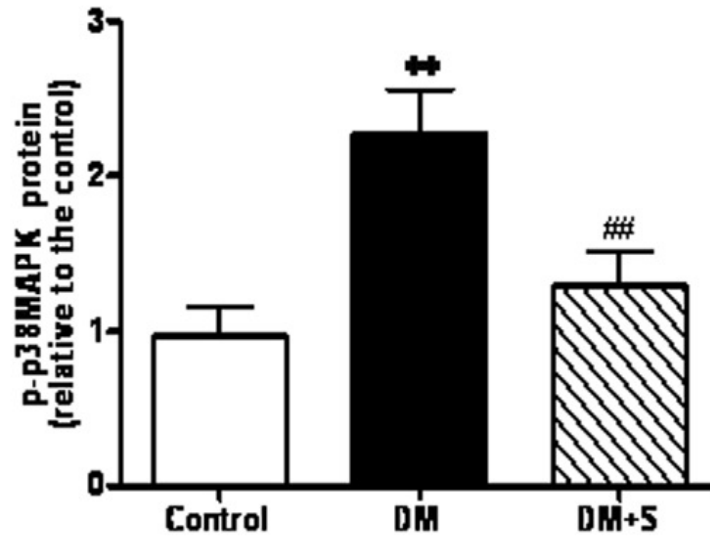
Fig. 2G





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