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Hypoxia-Mediated Mitochondrial Stress in RAW264.7 Cells Induces Osteoclast-Like TRAP-Positive Cells

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Abstract

Previously we showed that mitochondrial dysfunction induced by mitochondrial DNA depletion or treatment with electron transport chain inhibitors triggers a stress signaling involving activation of calcineurin and Ca^{2+}-responsive factors. In this study we show that exposure of RAW 264.7 cells to hypoxia, causing increased reactive oxygen species (ROS) production and disruption of mitochondrial transmembrane potential, also induced a similar stress signaling. Hypoxia caused increased $[Ca^{2+}]c$, activation of cytosolic calcineurin and induced expression of Ryanodine Receptor 2 (RyR2) gene. Prolonged hypoxia (5% O_2 for 5–6 days) also induced the expression of calcitonin receptor at high levels, and those of cathepsin K, and tartarate-resistant alkaline phosphatase (TRAP) at low-moderate levels in macrophage cells. Addition of RANKL had an additive effect suggesting different mechanisms of activation. Consistent with this possibility, prolonged hypoxia induced the formation of TRAP-positive osteoclast-like cells suggesting the occurrence of an autocrine mechanism for osteoclastogenesis.

Keywords

mitochondrial ROS production; stress signaling; macrophage differentiation; osteoclast marker gene expression

INTRODUCTION

The “retrograde response” is one mechanism by which cells respond to altered mitochondrial function. The signaling mechanism involved in retrograde response has been widely studied in both yeast and mammalian cells.[1] While the yeast response occurs mainly by the activation of the RTG pathway,[1,2] mammalian cell response involves more complex signaling. Mitochondrial dysfunction due to partial or complete depletion of mitochondrial DNA (mtDNA) triggers a specific mitochondrial stress signaling in C2C12 skeletal myoblasts, A549 lung carcinoma cells, and others.[3-7] This is characterized by elevated cytosolic calcium leading to activation of calcineurin-dependent NF-κB pathway. The mitochondrial stress-induced NF-κB pathway differs from the established canonical and noncanonical pathways.[8-10] The mitochondrial stress signaling pathway involves the release

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of p50:cRel heterodimer from IκBβ in the cytosol through calcineurin-mediated dephosphorylation of IκBβ. Activation of this stress signaling in C2C12 and A549 cells induces them to form invasive phenotypes.[3]

Hypoxic environment is a very common condition during fracture, inflammation, arthritis, and infection.[11] Hypoxia is also known to activate bone marrow precursor cells of the macrophage lineage.[12] RAW 264.7 cells differentiate into osteoclasts in response to receptor activator of NF-κB ligand (RANKL). Many of the signaling cascades activated during RANKL-stimulated osteoclastogenesis are mediated through reactive oxygen species (ROS).[13–16] Interestingly, a number of signaling factors activated under mitochondrial stress signaling are the same as those of osteoclastogenesis. We therefore set out to determine whether hypoxia triggers mitochondrial stress signaling in cultured macrophages and if the signaling promotes the differentiation of RAW 264.7 cells. We show here that long-term hypoxia induces the expression of many of the marker genes of osteoclastogenesis in RAW 264.7 cells and also augments the RANKL-induced differentiation.

MATERIALS AND METHODS

Cell Culture

RAW 264.7 mouse monocyte macrophages were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum. Cells at 70–80% confluence were grown under hypoxic conditions (0.5% O_2) for 10 h or normoxic conditions (21% O_2) for 10 h. Ascorbate, when used was added to cells at a final concentration of 500μM before starting hypoxia. In case of Rotenone (1μM) and mitoQ (10μM) treatment, the compounds were added to the medium during the last 4 h of hypoxia.

Measurement of ROS Production

Mitochondrial ROS production was measured by DCF fluorescence with modifications as described before.[17] Freeze-thawed and disrupted mitochondria (10 μg) from normoxic, hypoxic, and rotenone-treated cells were taken in 1 mL H-medium containing 5 μg of esterase fraction. Electron transport was initiated by adding 1 μM NADH. The fluorescence was recorded afteradding 1 μM DCFH-DA using a LPS-220B spectrofluorometer from Photon Technology International (Birmingham, NJ) at an excitation at 488 nm and emission at 525 nm for 25 min.

Measurement of Mitochondrial Membrane Potential

Membrane potential was measured by the oxidation of mitotracker orange by a spectrofluorometric assay as described before.[4] The membrane potential was measured as a function of mitochondrial uptake of MitoTracker Orange, CM-H2TM ROS (50 nM) (Invitrogen-Moleculor Probes, Eugene, OR) added to the cell suspension. Fluorescence was monitored for 20 min in a multiwavelength-excitation dual wavelength-emission Delta RAM PTI (Birmingham, NJ) spectrofluorometer at 525 nm excitation and 575 nm emission. CCCP (1 μM) was used as a positive control for disrupting the membrane potential.

Measurement of Cytosolic Calcium

$[Ca^{2+}]c$ levels were measured using Fura 2 fluorescence as described before.[4,18] Fluorescence was monitored at excitation 340/380 nm and emission at 510 nm. Calibration of Fura 2FF/FA signal was carried out as in the Delta RAM PTI suggested protocol using a calibration buffer containing 10 mM EGTA-Tris-HEPES, pH 8.5, and 5 mM NaCl.

Cell Fractionation and Immunoblot Analysis

Cells were homogenized in a cocktail of 300 mM sucrose, 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 5 mM MgCl2, 1 mM orthovanadate, 10 μM molybdic acid, 10 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and complete protease inhibitor mix (Roche Molecular Biochemicals, Indianapolis, IN) in a Dounce homogenizer and fractionated into nuclear, postmitochondrial, and mitochondrial fractions as described before.[19] Proteins resolved on polyacrylamide gels were subjected to immunoblot analysis using antibodies. The immunoblots were developed using the Pierce Super (Rockford, IL) signal West Femto maximum sensitivity substrate kit, imaged and quantified in a Bio-Rad (Hercules, CA) Fluor-S imaging system.

Calcineurin activity was assayed according to Antoni *et al.*[20] with modifications.[21] Total serine/threonine phosphatase activity was first determined by incubating cell lysate (2 μg) with threonine phosphopeptide (Upstate, Cat 12-219; Lake Placid, NY) in a final volume of 25 μL for 20 min at 30°C in 50 μM Tris-Cl, pH 7.0, 0.1 mM $CaCl_2$. The released free phosphate was measured by incubating with 100 μL of malachite green and measuring the absorbance at 660 nm after 15 min. In parallel, control incubations containing phosphatase inhibitors mix I (NaF, 5mM; okadaic acid, 500 nM; orthovanadate, 100 μM; FK506, 1 nM; calyculin, 0.1 μM and cyclosporine A, 100 μM) and phosphatase inhibitors mix II (FK506, 1nM; calyculin, 0.1 μM; and cyclosporine A, 100 μM) were also carried out. Nonspecific phosphatase activity obtained from incubation with phosphatase inhibitors mix I was subtracted from total serine/threonine phosphatase activity to obtain specific serine/threonine phosphatase activity. Calcineurin activity was calculated by subtracting the activity obtained from incubation with phosphatase inhibitors mix II from specific serine/threonine phosphatase activity.

mRNA Quantitation by Real-Time PCR

Total RNA was isolated using Trizol reagent as per supplier's protocol (Invitrogen, Carlsbad, CA). cDNA was generated from 5 mg RNA using the cDNA Archive kit from Applied Biosystems (Foster City, CA) and 50 ng of this cDNA was used as template per reaction. Relative quantification of calcitonin receptor (CTR), cathepsin K (CatK), tartrate-resistant acid phosphatase (TRAP), and GLUT 4 mRNA by real-time PCR was done using SYBR Green (Applied Biosystems) in an ABI PRISM 7300 sequence detection system (Applied Biosystems). Data were normalized to β-actin (for SYBR green) as endogenous controls.

TRAP Staining

TRAP staining was performed using the Sigma (St. Louis, MO) kit according to the manufacturer's instructions. RAW 264.7 cells at 30–40% confluence were maintained at 5% O_2 for 5–6 days. Fresh medium equilibrated at 5% O_2 was replaced every 2 days. At the end of hypoxia, the adherent cells were fixed with 50% (v/v) ethanol/PBS for 10 min, fixed again with ethanol–acetone (50:50, v:v) for 1 min, and incubated for 10 min at room temperature with the TRAP-staining solution. Cells that stained dark red were counted as TRAP-positive cells.

RESULTS

Hypoxia-Induced Mitochondrial ROS Generation and Disruption of Transmembrane Potential

Mitochondrial ROS in cells subjected to hypoxia was measured using the modified DCFHDA method.[17] RAW 264.7 cells maintained under hypoxia for 10 h produced higher fluorescence than corresponding normoxic cells (FIG. 1A). Mitochondrial origin of these reactive species is seen by decrease in DCF fluorescence in cells treated with rotenone (1 μM), an inhibitor of complex I. Although not shown, ascorbate, and inhibitors of complex I and III (antimycin and myxothiazol) also inhibited DCF fluorescence.

Membrane potential ($\Delta\psi$ m) was measured using mitotracker orange. As shown in FIGURE 1B normoxic cells exhibited a steady increase in the fluorescence over a period of 20 min of incubation with mitotracker orange.[4] Hypoxic cells on the other hand, showed significantly lower mitotracker fluorescence. As a positive control for disruption of membrane potential, cells were incubated with CCCP, a mitochondrial ionophore. As seen in FIGURE 1B, CCCP treatment completely abolished membrane potential.

Hypoxia Causes Elevated Cytosolic Calcium and Calcineurin Activity

Cytosolic Ca^{2+} homeostasis is known to be actively influenced by mitochondria and other intracellular Ca^{2+} stores. Earlier reports have shown that disruption of membrane potential in mtDNA-depleted C2C12 cells causes a sustained increase in the cytosolic-free calcium levels.[4,5] This increase was in parallel with higher expression of the calcium channel, type 1 ryanodine receptor (RYR1). As shown in FIGURE 2A cells grown under hypoxia exhibited increased $[Ca^{2+}]c$ compared to normoxic controls. Although not shown, treatment with ascorbate or rotenone reduced the Ca^{2+} level to normoxic levels. Caffeine is an agonist of RYR calcium channels. In normoxic cells caffeine treatment had no effect on fura 2 fluorescence, while the agonist induced Ca^{2+} release in cells subjected to hypoxia (FIG. 2A). Acetylcholine, an agonist for IP3 Ca^{2+} channel caused a more marked Ca^{2+} release than in normoxic controls. These results show that hypoxia-induced mitochondrial dysfunction causes a change in cellular Ca^{2+} homeostasis similar to that reported in mtDNA-depleted cells.[4,5]

Consistent with increased caffeine-mediated Ca^{2+} release, immunoblot in FIGURE 2B shows that RYR2 protein level is increased by >2.5-fold in hypoxiagrown cells. Although not shown, treatment with rotenone and ascorbate markedly reduced the level of RyR2

protein suggesting a role for mitochondrial stress in induced steady-state levels of the protein.

It is also seen from FIGURE 3 that calcineurin activity was increased nearly fourfold in macrophages grown under hypoxia. Rotenone markedly reduced the activity and as expected FK506 inhibited the activity by >80%. These results suggest that mitochondrial ROS production and possibly ROS-induced change in membrane potential plays a role in increased calcineurin activity during hypoxia.

Hypoxia Induces the Expression of Marker Genes for Osteoclastogenesis

It is known that macrophage cells differentiate into osteoclasts upon activation of its RANK receptor by its specific ligand, RANKL expressed by osteoblasts. The RANKL expression in osteoblasts in turn is mediated by cytokines and also by ROS.[22] We therefore measured the mRNA levels of some of the marker proteins upregulated during osteoclastogenesis in hypoxia-grown macrophages. mRNA levels of CatK, TRAP, and CTR were measured in normoxic and hypoxic cells. FIGURE 4A shows that the level of calcitonin receptor was increased several folds under hypoxia, and addition of RANKL did not further increase the mRNA level. The levels of the other two markers, CatK (FIG. 4B) and TRAP (FIG. 4C) were only marginally increased by hypoxia. Addition of RANKL induced mRNA levels by threefold in both cases. Notably, hypoxia and RANKL together induced the mRNA levels by over fourfold suggesting an additive effect in both cases.

Formation of TRAP-Positive Cells by Hypoxia-Mediated Mitochondrial Stress in RAW 264.7 Cells

Macrophages grown under normoxia and hypoxia (5% O_2, for 5 days) were stained for TRAP expression. The TRAP-positive cells that differentiate into-osteoclasts stain red. As seen from FIGURE 5, extended exposure to mild hypoxia resulted in the formation of a small population of TRAP-positive cells. Addition of RANKL induced > sixfold increased TRAP-positive cells. Interestingly, hypoxia and RANKL treatment together had an additive effect in that > eightfold higher levels of TRAP-positive cells were observed. These results suggest an intriguing possibility that hypoxia and RANKL induce osteoclas-togenesis by different mechanisms.

DISCUSSION

It is widely known that cells generate excess of ROS during hypoxic conditions above and beyond the level, which cannot be managed by the cellular antioxidant defenses. Mitochondrial electron transport chain (ETC) is a major source of ROS both under normoxic and hypoxic conditions. Complex I and III have been suggested to be the major source of ROS although other membrane complexes and matrix enzymes also produce ROS, albeit, at lower levels.[17,23] Mitochondrially generated ROS is known to cause damage to the ETC complexes, increased lipid peroxidation, inactivate TCA cycle enzymes and eventually cause the disruption of mitochondrial transmembrane potential. In this study we show for the first time that hypoxia induces mitochondrial stress signaling similar to that observed in partially depleted or completely depleted mtDNA (ρ° cells) cells through increased $[Ca^{2+}]c$

and activation of calcineurin. Although not shown hypoxia-induced stress signaling also activates NF-κB and other stress specific signature factors and induced expression of nuclear target genes. RyR family genes (RyR1, RyR2 and RyR3) in different cells are the prototype genes affected by the stress signaling.[4,5] In keeping with this, hypoxia-induced mitochondrial stress also induced the expression of RyR2 in macrophages.

RAW 264.7 macrophages are known to differentiate into osteoclasts when stimulated by RANKL. In the physiological environment, osteoblasts produce RANKL which binds to RANK expressed on the surface of osteoclast precursors and initiates differentiation.[24] The signaling pathways of osteo-clastogenesis have been extensively studied. Many recent reports have shown that hypoxia and H_2O_2 are major stimulators of osteoclast activity.[25–27] Hypoxia is also shown to be a stimulator of activation of cells derived from bone marrow precursors.[12] It is known that active pathological bone destruction occurs at sites with low pO .[25] 2 Macrophages encounter low pO_2 under different pathological conditions including arthritis, infection, fracture and ischemia.[11] Recent reports show that the activity of RANKL in inducing osteoclastogenesis in macrophages is mediated by ROS.[13,28] It has been shown that JNK, p38, and NF-κB activation that occur during osteoclastogenesis upon RANKL stimulation are mediated through ROS generated by Nox1 and mitochondrial ETC.[28] RANKL expression and excretion in osteoblasts is known to occur in response to cytokines, and/or, ROS production.[22]

Our results show that hypoxia-mediated stress activates some of the key mediators of osteoclastogenesis like calcineurin, NF-κB, C/EBP δ, and NFAT (results not shown). Interestingly, under moderate but prolonged hypoxia (5–6 days) prevalent in arthritis, and other pathological conditions, important marker genes of osteoclasts like CatK, TRAP, CTR, and MMP9 are induced. An interesting observation is that the levels of hypoxia-inducible and RANKL-inducible CatK and TRAP expression are additive. These results suggest that ROS produced during hypoxic stress induces the expression of a number of osteoclastogenesis markers most likely by a mechanism not involving RANKL. In support of this possibility, prolonged hypoxia induced the formation of osteoclast-like TRAP-positive cells in a pure population of RAW 264.7 cells. These results suggest the possible occurrence of an autocrine mechanism for the differentiation of osteoclasts during prolonged hypoxic conditions.

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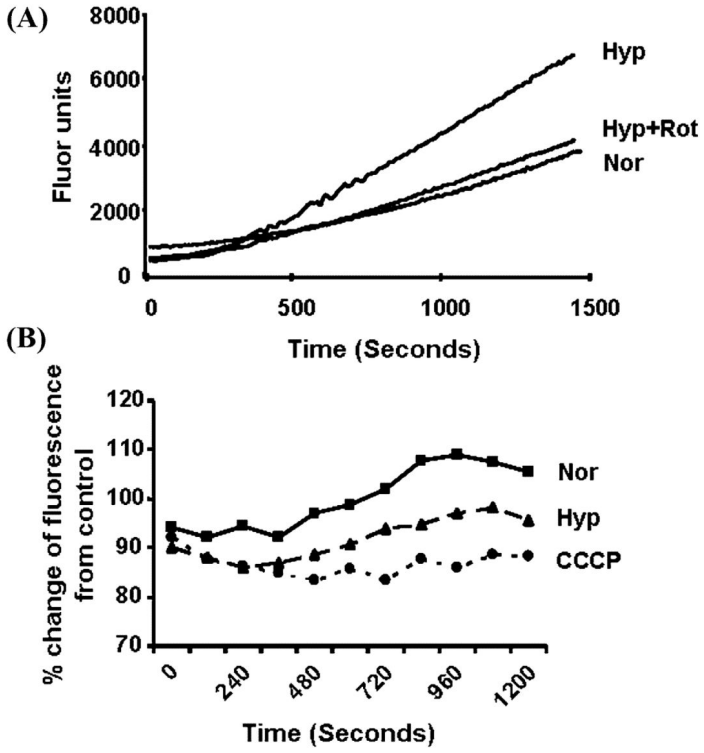

FIGURE 1.

Measurement of ROS production and mitochondrial membrane potential. (**A**) Rate of fluorescence emission by incubating mitochondria with DCFHDA. Freeze-thawed mitochondria (25 μg) from normoxic (Nor), hypoxic (Hyp), and from hypoxic cells treated with 1 μM rotenone (Hyp+Rot) were incubated with DCFHDA (1 μM) and partially purified cytosolic protein fraction rich in esterase activity (10 μg/mL). Fluorescence emission was measured at 525 nm after excitation at 488 nm. (**B**) Spectrofluorometric patterns of mitochondrial uptake of MTO (50 nM) in normoxic (Nor) and hypoxic (Hyp) cells as described in “Materials and Methods.” CCCP-treated cells were used as positive control for membrane potential disruption. Excitation at 525 nm and emission at 575 nm were followed.

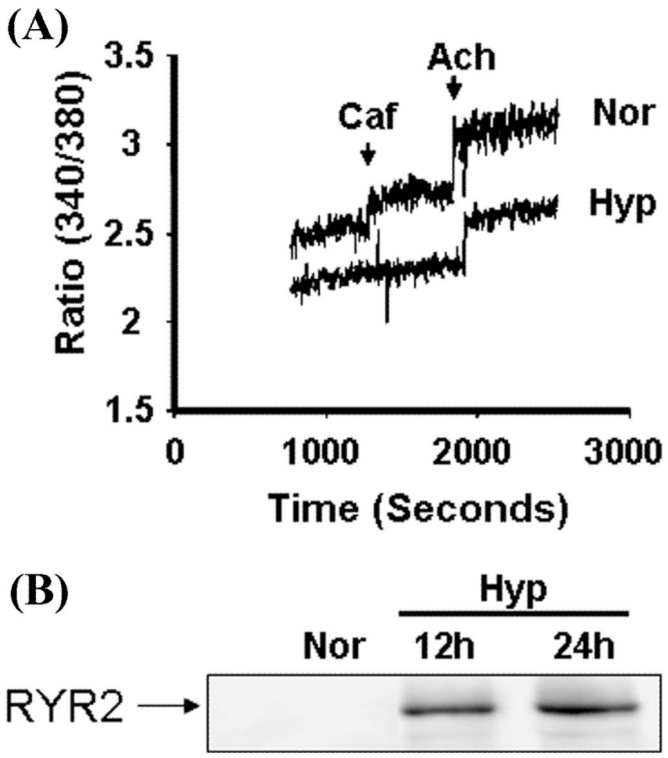

FIGURE 2.

Steady-state $[Ca^{2+}]c$ and agonist-induced $[Ca^{2+}]$ release in hypoxic cells. (**A**) Spectrofluorometric measurement of $[Ca^{2+}]$ release patterns in normoxic (Nor) and hypoxic (Hyp) cells in response to 20 mM caffeine (Caf) and 10 mM acetylcholine (Ach). (**B**) Immunoblot analysis of 100 μg protein from postnuclear fractions of normoxic and hypoxic cells with antibody to RyR2 channel protein (C3-33 Sigma).

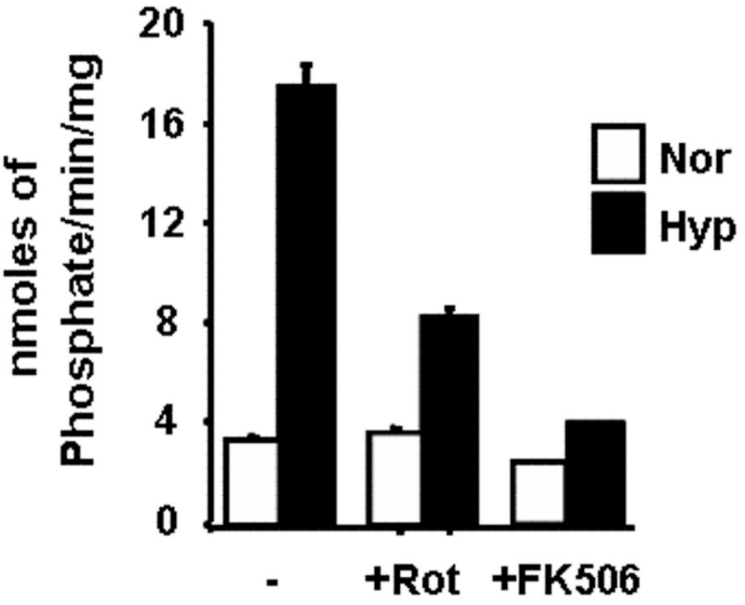

FIGURE 3.

Measurement of calcineurin activity. Calcineurin activity was measured in the cytosolic fraction (2 μg) of normoxic and hypoxic cells. Rotenone (1 μM) and FK506 (10 nM) were added to the cells during the last 4 h of hypoxia. Calcineurin-specific phosphatase activity was calculated as described in the “Materials and Methods” section.

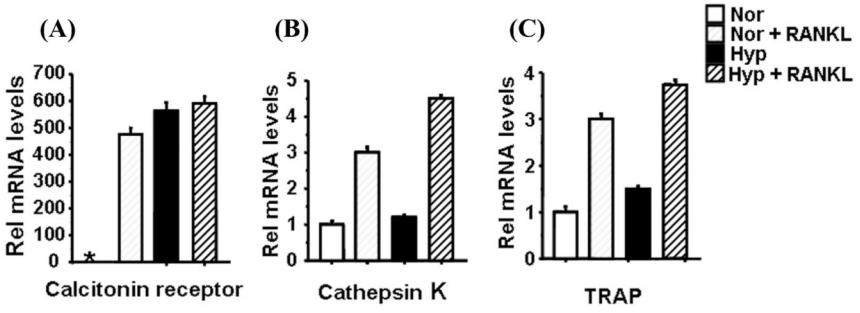

FIGURE 4.

Measurement of osteoclast marker gene expression by real-time PCR. mRNA was prepared from normoxic, hypoxic, and RANKL (10 ng/mL)-treated cells by Trizol method. mRNA levels for (**A**) calcitonin receptor, (**B**) Cathepsin K, and (**C**) tartrate-resistant acid phosphatase (TRAP) were quantitated by real-time PCR using SYBR green dye and β actin as endogenous control. *Reflects value (=1).

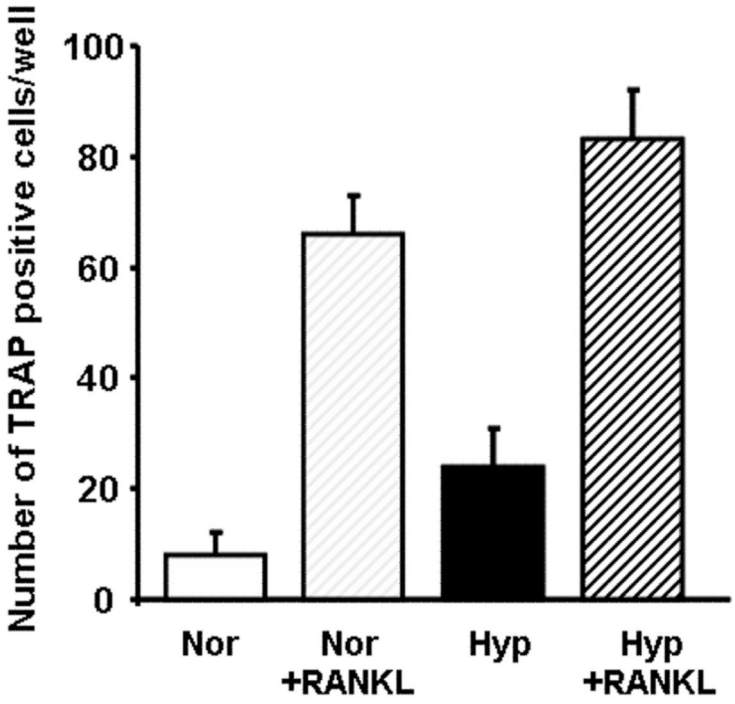

FIGURE 5.

Measurement of TRAP-positive RAW 264.7 cells. RAW 264.7 cells were plated in 96-well tissue culture plates at a density of 1×10^3 cells/well, and cultured with or without RANKL (10 ng/mL) under normoxia or hypoxia for 6 days. Cells were stained for TRAP using Sigma Kit and the TRAP-positive cells were counted. Values represent the means ± SD (n = 3). All data are representative of three different experiments.