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Signal Transduction of Opioid-induced Cardioprotection in Ischemia–Reperfusion

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Background: **Morphine reduces myocardial ischemia–reperfusion injury *in vivo* and *in vitro*. The authors tried to determine the role of opioid δ_1 receptors, oxygen radicals, and adenosine triphosphate–sensitive potassium (K_{ATP}) channels in mediating this effect.**

Methods: **Chick cardiomyocytes were studied in a flow-through chamber while pH, flow rate, oxygen, and carbon dioxide tension were controlled. Cell viability was quantified by nuclear stain propidium iodide, and oxygen radicals were quantified using molecular probe 2',7'-dichlorofluorescin diacetate.**

Results: **Morphine (1 μM) or the selective δ-opioid receptor agonist BW373U86 (10 pM) given for 10 min before 1 h of ischemia and 3 h of reoxygenation reduced cell death (31 ± 5%, n = 6, and 28 ± 5%, n = 6 [$P < 0.05$], respectively, 53 ± 6%, n = 6, in controls) and generated oxygen radicals before ischemia (724 ± 53, n = 8, and 742 ± 75, n = 8 [$P < 0.05$], respectively, *vs.* 384 ± 42, n = 6, in controls, arbitrary units). The protection of morphine was abolished by naloxone, or the selective δ_1-opioid receptor antagonist 7-benzylidenenaltrexone. Reduction in cell death and increase in oxygen radicals with BW373U86 were blocked by the selective mitochondrial K_{ATP} channel antagonist 5-hydroxydecanoate or diethyldithiocarbamic acid (1000 μM), which inhibited conversion of O_2^- to H_2O_2. The increase in oxygen radicals was abolished by the mitochondrial electron transport inhibitor myxothiazol. Reduction in cell death was associated with attenuated oxidant stress at reperfusion.**

Conclusion: **Stimulation of δ_1-opioid receptors generates oxygen radicals *via* mitochondrial K_{ATP} channels. This signaling pathway attenuates oxidant stress and cell death in cardiomyocytes.**

ACTIVATION of opioid receptors decreased morbidity in mice after hypoxia[1] and reduced the size of myocardial infarct in anesthetized rats.[2] Morphine has cardioprotective effects in cardiomyocytes.[3] We used chick ventricular cardiomyocytes in a model of simulated ischemia–reoxygenation to determine the role of δ_1-opioid receptors, oxygen radicals, and mitochondrial adenosine triphosphate–sensitive potassium (K_{ATP}) channels in mediating the reduction of cardiocyte death with morphine. We also sought the source and regulation of the oxygen radicals generated. For this purpose, we used the nonselective opioid receptor antagonist naloxone, the selective δ-opioid receptor agonist BW373U86,[4,5] the antagonist 7-benzylidenenaltrexone (BNTX),[6] and the mitochondrial selective K_{ATP} channel antagonist 5-hydroxydecanoate (5-HD).[7]

Free radicals are important in the pathogenesis of injury after myocardial ischemia and reperfusion.[8–10] Stimulation of signal transduction by opioid receptors may attenuate oxidant stress in ischemia–reperfusion, thus reducing cell death. Few studies have attempted direct quantification of free radicals during ischemia and reperfusion, but rather rely on observations of the behavior of free radical scavengers. We monitored free radical generation continuously throughout ischemia and reperfusion.

Materials and Methods

Cardiomyocyte Preparation

Embryonic chick ventricular myocytes were prepared according to a method described by Barry *et al.*[11] and modified by Vanden Hoek *et al.*[12] Ten-day-old embryonic hearts were collected and placed in a balanced salt solution lacking calcium and magnesium (Life Technologies Inc., Grand Island, NY). The ventricles were then minced, and the myocytes were dissociated by use of four to six repeats of trypsin (0.025%, Life Technologies, Inc.) degradation at 37°C with light agitation. Isolated cells were transferred to a solution with trypsin inhibitor for 8 min, filtered through a 100-μm mesh, centrifuged for 5 min at 1,200 rpm at 4°C, and resuspended in a nutritive medium described by Chandel *et al.*[13] and Duranteau *et al.*[14] The resupended myocytes were placed on Petri dishes in a humidified incubator (5% CO_2, 95% air at 37°C) for 45 min to promote adherence of fibroblasts. Nonadherent cells were counted with a hemocytometer, and cell viability was measured with trypan blue (0.4%). Approximately 1×10^6 cells were pipetted onto coverslips (25 mm) and incubated for 3–4 days until synchronous contractions of the monolayer were visible. Tests were performed on the spontaneously beating cells on day 3 or 4 after isolation.

Perfusion System

Glass coverslips containing spontaneously contracting embryonic chick myocytes were placed in a stainless steel flow-through chamber (1-ml volume; Penn Century Co., Philadelphia, PA). To minimize the oxygen exchange between the chamber wall and the perfusate, the chamber was sealed with gaskets. The chamber was then placed onto a temperature-controlled platform (37°C) on an inverted microscope. A water-jacketed glass equilibration column, mounted higher than the

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microscope stage, equilibrated the perfusate to the desired oxygen tensions. A buffered salt solution served as the standard perfusion media (117 mM NaCl, 4.0 mM KCl, 18 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 1.21 mM $CaCl_2$, and 5.6 mM glucose), which was equilibrated for 1 h before the experiment by bubbling with a gas mixture of 21% oxygen, 5% carbon dioxide, and 74% nitrogen. A buffered salt solution containing no glucose with 2-deoxyglucose (20 mM) added to inhibit glycolysis was bubbled with a gas mixture of 20% carbon dioxide and 80% nitrogen for 1 h before ischemia. Stainless steel or polymer tubing with low oxygen solubility connected the glass equilibration column to the flow-through chamber to minimize ambient oxygen transfer into the perfusate. In previous studies, low levels of oxygen tension in the chamber were confirmed during conditions identical to those in experiments that used an optical phosphorescence quenching method.[15,16]

Determination of Cell Viability

An inverted microscope, equipped for epifluorescent illumination, included a xenon light source (75 W), a shutter and filter wheel, a 12-bit digital cooled camera, and appropriate excitation and emission filter tubes. The microscope also was equipped with Hoffman-modified phase illumination to accentuate surface topology, facilitating the measurement of contractile motion. Fluorescent cell images were obtained with an ×10 objective lens. Data were acquired and analyzed with Metamorph software (Boston, MA). Cell viability was quantified with the nuclear stain propidium iodide (5 μM, Molecular Probes, Eugene, OR), an exclusion fluorescent dye that binds to chromatin on loss of membrane integrity.[17] Propidium iodide is not toxic to cells over a course of 8 h, permitting its addition to the perfusate throughout the experiment. To facilitate the completion of the experiment, digitonin (300 μM) was added to the perfusate for 1 h. Digitonin disrupts the integrity of all cell membranes, allowing propidium iodide to enter cells so that the maximum propidium iodide value is obtained. Percent loss of viability (cell death) was then expressed relative to the maximum value after 1 h of digitonin exposure (100%).

Measurement of Free Radicals

Free radical generation in cells was assessed using the probe 2',7'-dichlorofluorescin (DCFH). The membrane-permeable diacetate form of DCFH, DCFH-DA, was added to the perfusate at a final concentration of 5 μM. Once in the cell, esterases cleave the acetate groups on DCFH-DA, thus trapping DCFH intracellularly.[18] Free radicals in the myocytes oxidize the DCFH, yielding the fluorescent product DCF.[19] DCFH is readily oxidized by H_2O_2 or hydroxyl radical but is relatively insensitive to superoxide.[12,14] Fluorescence was measured with an excitation wavelength of 480 nm, dichroic 505-nm long pass, and emitter bandpass of 535 nm with neutral density filters to attenuate the excitation light intensity. Fluorescence intensity was assessed in clusters of several cells identified as regions of interest. Background was identified as an area without cells or with minimal cellular fluorescence. Intensity values are reported as the percentage of initial values after subtraction of the background value.

Chemicals

Morphine sulfate was purchased from Elkins Sinn, Inc. (Cherry Hill, NJ). Diethyldithiocarbamic acid (DDC), BW373U86, and 5-HD were purchased from Sigma Chemical Co. (St. Louis, MO). Naloxone was purchased from Research Biochemical International (San Diego, CA). BNTX was obtained from Toray Industries, Inc. (Kanagawa, Japan). Morphine sulfate, 2-mercaptopropionyl glycine (2-MPG), naloxone, BNTX, DDC, or 5-HD were dissolved in buffered salt solution before administration. Propidium iodide, myxothiazol, and DCFH-DA were purchased from Molecular Probes (Eugene, OR).

Experimental Design

Eleven groups of cardiomyocytes (control, morphine, BW373U86, naloxone, naloxone+morphine, BNTX, BNTX+morphine, DDC, DDC+BW373U86, 5-HD, and 5-HD+BW373U86) were studied. Cells were subjected to 60 min of ischemia before 3 h of reoxygenation. Saline (control series), morphine (1 μM), or BW373U86 was added to the perfusate for 10 min in treated cells followed by 10 min of a drug-free period. The other cells were treated with naloxone (10 μM), BNTX (0.1 μM), DDC (1 mM), or 5-HD (100 μM) in perfusate during the hour of baseline before 60 min of ischemia.

Additional studies (with saline, morphine, BNTX, BNTX+morphine, BW373U86, DDC, DDC+BW373U86, myxothiazol, myxothiazol+BW373U86, 5-HD, 5-HD+BW373U86) were performed to examine the role of δ_1-opioid receptors, mitochondrial K_{ATP} channels, and the mitochondrial electron transport system in regulating oxygen radicals. The doses of various antagonists were chosen on the basis of preliminary studies[20] that showed that these drugs alone had no significant effects on baseline free radical generation compared with controls. Antagonists used in this study were infused during the first 60-min period before the prolonged simulated ischemic period.

Statistical Analysis

Data are expressed as mean ± SEM. Differences between groups for cell death and free radical production were compared by a two-factor analysis of variance and the Fisher least significant difference test. Return of contractile function was analyzed by the Fisher exact test. Differences between groups were considered significant at a value of $P < 0.05$.

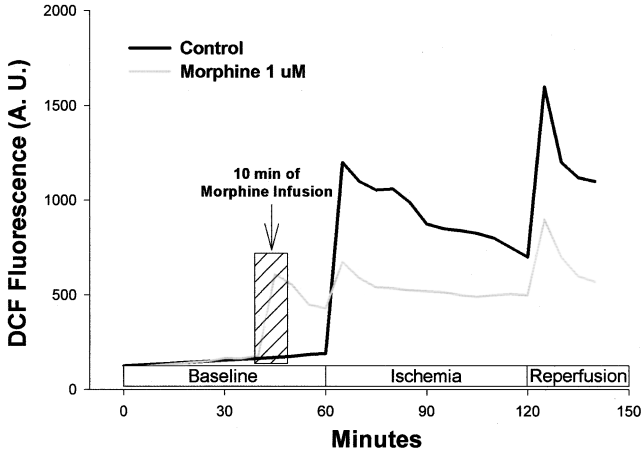

Fig. 1. Graph showing one representative experiment from control and morphine-treated groups. DCF = dichlorofluorescein; A.U. = arbitrary units.

Results

Opioids Generate Oxygen Radicals before Simulated Ischemia

Figure 1 documents one representative experiment from control and morphine-treated groups showing intensity of DCF fluorescence throughout the study.

Morphine or BW373U86 increased DCFH oxidation (an index of oxygen radical production) compared with controls (724 ± 53, n = 8, and 742 ± 75, n = 8 [$P < 0.05$] *vs.* 384 ± 42, n = 6, in controls, arbitrary units; fig. 2A). The increase in oxygen radicals with morphine was abolished by treatment with BNTX (377 ± 87, n = 7); BNTX alone had no effects on DCFH oxidation at baseline (437 ± 43, n = 3; fig. 2A).

The increase in oxygen radicals with BW373U86 was abolished by DDC (fig. 2B). The precursor of H_2O_2 is superoxide (O_2^-). Superoxide dismutase is an enzyme in cytosol that catalyzes the conversion of O_2^- to H_2O_2. DDC is a cytosol Cu, Zn-superoxide dismutase inhibitor that attenuates production of H_2O_2. DCFH is more readily oxidized by H_2O_2 than by superoxide.[21] Opioid-produced oxygen radicals are likely to be H_2O_2.

The increase in oxygen radicals with BW373U86 also was abolished by myxothiazol, a mitochondrial electron transport inhibitor, or 5-HD, a selective mitochondrial K_{ATP} channel blocker. Myxothiazol or 5-HD alone had no effects on baseline DCFH oxidation (figs. 2C and 2D).

Opioiods Reduce Cell Death

Morphine reduced cell death in ischemia–reperfusion (31 ± 5%, n = 6, *vs.* 53 ± 6%, n = 6; fig. 3A). The protection of morphine was abolished by the nonspecific opioid receptor antagonist naloxone or by the selective δ_1-opioid receptor antagonist BNTX (48 ± 7%, n = 4, and 58 ± 7%, n = 7). Naloxone or BNTX alone had no effect on cell death.

The δ-opioid receptor agonist BW373U86 produced the same protection afforded by morphine. DDC and 5-HD, which abolished the oxygen radicals produced by BW373U86 (fig. 2B), also blocked its protection (fig. 3B). DDC and 5-HD alone had no effects on cell death.

Opioids Attenuate Oxidant Stress

Morphine and BW373U86 markedly attenuated oxidant stress during ischemia (fig. 4) and reperfusion (fig. 5). Interruption of the signaling pathway with blockade of δ_1-opioid receptors, mitochondrial K_{ATP} channels, or oxygen radicals restored oxidant stress to a level indistinguishable from that in controls. These effects correlated with reduction in cell death.

Opioids Have No Effects when Administered during Simulated Ischemia–Reoxygenation

Morphine (1 μM) or BW373U86 (10 pM) had no effect on cell death when administered during ischemia–reperfusion (45 ± 6%, n = 3, 49 ± 7%, n = 3 *vs.* 53 ± 6%, n = 6).

Discussion

Our results show that transient administration of opioids reduces cell death by attenuating oxidant stress in isolated cultured cardiomyocytes. This study provides direct evidence that δ_1-opioid receptors, oxygen radicals, and mitochondrial K_{ATP} channels are important intracellular signals in mediating opioid protection.

The Role of δ_1-Opioid Receptors

Schultz *et al.*[2] were the first to show that stimulation of opioid receptors reduced the size of myocardial infarct in anesthetized rats. Functional opioid receptors exist in ventricular myocytes.[3,22] Morphine or BW373U86, a selective δ-opioid receptor agonist,[4,5] attenuated ischemia–reperfusion injury in isolated cultured cardiomyocytes. The protection conferred by morphine was abolished with the nonselective opioid receptor antagonist naloxone or the selective δ_1-antagonist BNTX,[6] as other investigators have shown.[3] The subtypes of receptors involved in the mechanism of action have not been established, although morphine has a high affinity for the μ-opioid receptor.[3,22] The cardioprotection of morphine appears to be δ_1-opioid receptor–mediated.

Free radicals are a contributing factor in the pathogenesis of myocardial injury after ischemia and reperfusion.[10,23] In our study, morphine and the δ-opioid receptor agonist BW373U86 markedly attenuated oxidant stress. These effects of morphine were reversed with the selective δ_1-opioid receptor antagonist BNTX. We previously showed that monophosphoryl lipid A reduced cardiac infarct size *via* a decrease of free radicals from neutrophils.[24] Thus, reduced cell death with opioids

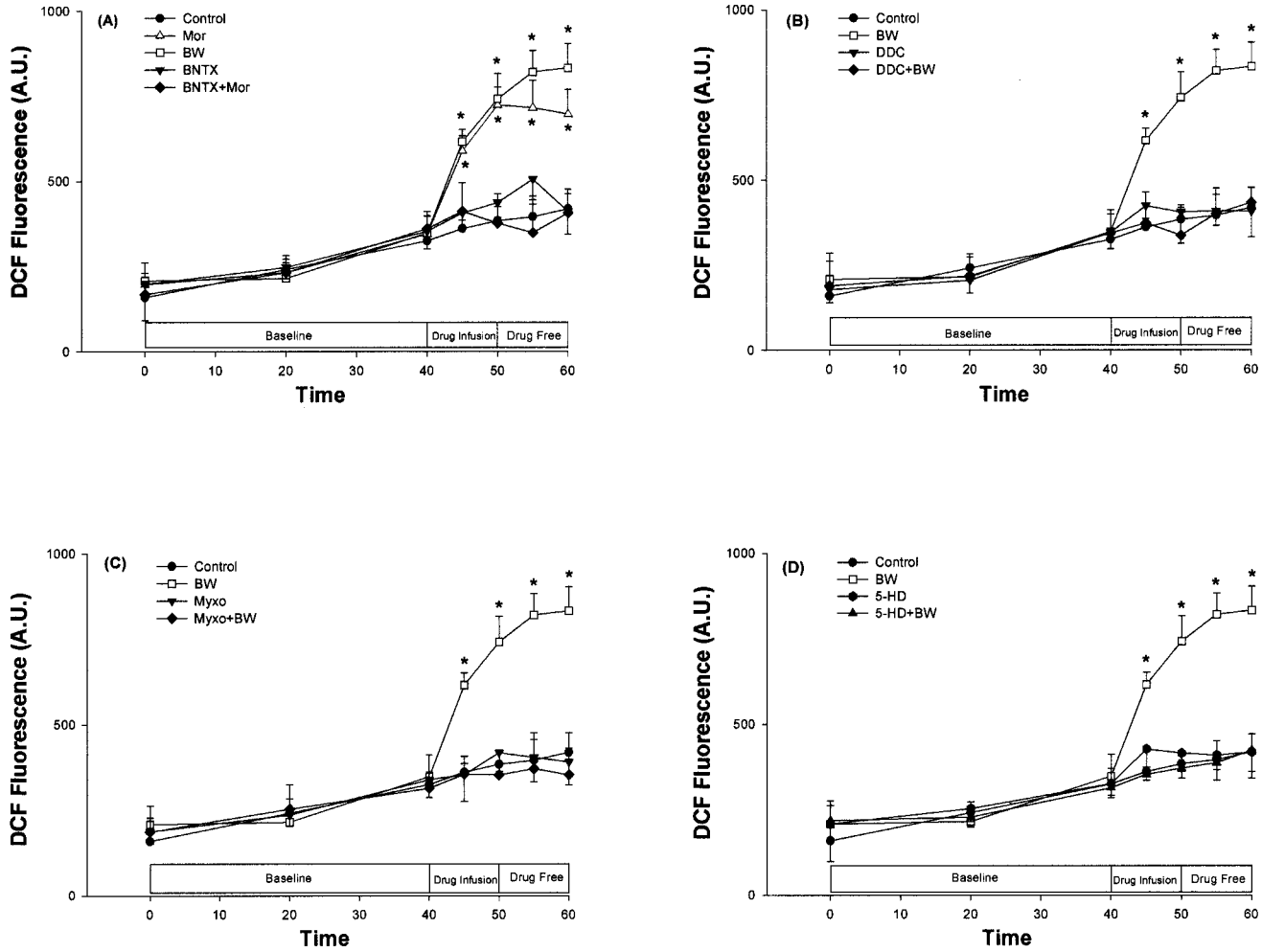

Fig. 2. In control cells, the intensity of 2',7'-dichlorofluorescin (DCF) fluorescence increased slightly during 1 h (Control). Infusion of morphine or BW373U86 for 10 min followed by 10 min of a drug-free period increased the intensity of DCF fluorescence (*A*). The increase in intensity with morphine was abolished by pretreatment with 7-benzylidenenaltrexone (BNTX+Mor). The increase in intensity with BW373U86 was abolished by pretreatment with diethyldithiocarbamic acid (DDC), an inhibitor for H_2O_2 production (DDC+BW; *B*); myxothiazol (Myxo+BW; *C*); or 5-hydroxydecanoate (5-HD+BW; *D*). *$P < 0.05$. A.U. = arbitrary units.

correlates with lessened oxidant stress. δ_1-Opioid receptors are important in these effects.

Role of Oxygen Radicals

How direct stimulation of δ_1-opioid receptors reduces ischemia–reperfusion injury is unknown. Transient administration of morphine or the δ-opioid receptor agonist BW373U86 increased oxygen radicals before the start of ischemia. The increase was abolished by naloxone or the selective δ_1-opioid receptor antagonist BNTX. These results indicate that δ_1-opioid receptor stimulation increases intracellular oxygen radicals. The radicals before simulated ischemia trigger the cardioprotective signal transduction.

An increase in oxygen radicals (trigger) correlates with reduced cell death. Both effects were abolished with DDC, an inhibitor of superoxide dismutase that catalyzes conversion of superoxide to hydrogen peroxide (H_2O_2). Thus, H_2O_2 seems to be a major component of opioid-induced oxygen radicals. Biologic oxidants regulate intracellular signal transduction.[25,26] Oxygen radicals are intracellular second messengers in hypoxia, ischemia, and acetylcholine-mediated cardioprotection in cardiocytes.[14,20,25] Our results and those of other investigators[3,22,25,26] indicate that stimulation of δ_1-opioid receptors produces oxygen radicals (trigger), mainly H_2O_2, mediating cardioprotective effects of opioids.

Source of the Oxygen Radicals

The increase in oxygen radicals (trigger) with activation of δ_1-opioid receptors was attenuated by myxothiazol, a mitochondrial site III electron transport inhibitor. Mitochondria are the source of oxygen radicals produced by hypoxic preconditioning[27] and seem to be the source of opioid-produced oxygen radicals. Pretreatment with 5-HD, a selective mitochondrial K_{ATP} channel antagonist, prevented the production of radicals by BW373U86. Thus, opening mitochondrial K_{ATP} channels

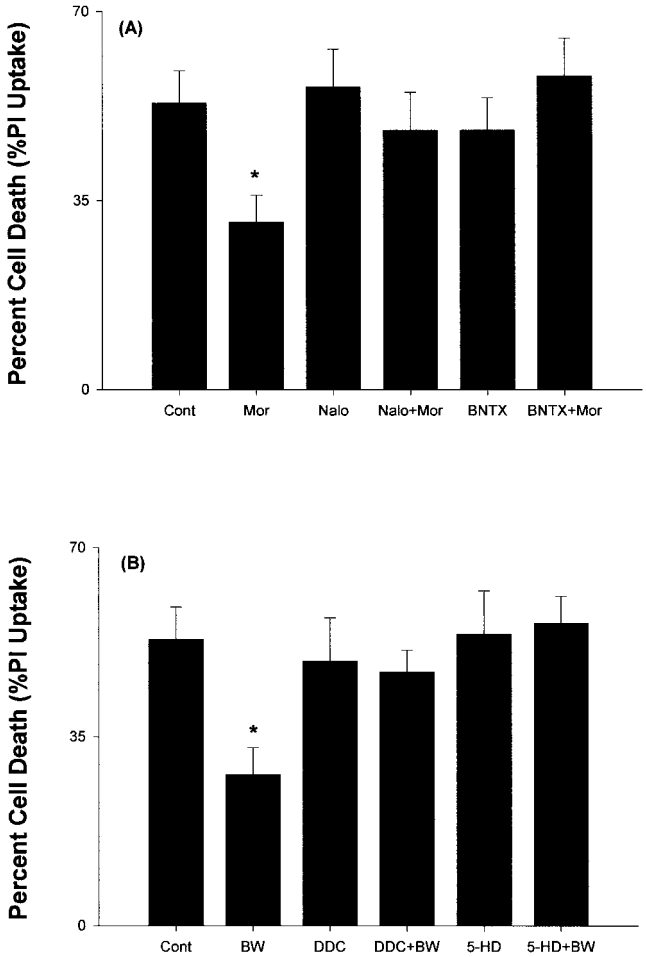

Fig. 3. Effects of opioids on cell death as assessed by propidium iodide (PI) uptake. Cardiomyocytes, equilibrated at normoxic conditions for 40 min (baseline), were subjected to infusion for 10 min of morphine or BW373U86, followed by 10 min of a drug-free period. Control cells were equilibrated at normoxic conditions for 60 min (Cont). All cells were subjected to 60 min of ischemia and 3 h of reoxygenation. Morphine (Mor) or BW373U86 significantly reduced cell death compared with controls. Treatment with naloxone (Nalo+Mor) or 7-benzylidenenaltrexone (BNTX+Mor) abolished the protection of morphine (*A*). The protection of BW373U86 was abolished by diethyldithiocarbamic acid (DDC), an inhibitor for H_2O_2 production (DDC+BW) or 5-hydroxydecanoate (5-HD+BW; *B*). *$P < 0.05$.

plays a role in the formation of oxygen radicals. Activation of mitochondrial K_{ATP} channels (trigger) was important in acetylcholine-induced oxygen radicals in isolated cardiomyocytes.[20] K_{ATP} channel activation is an intermediate step after δ_1-opioid receptor stimulation. In addition, reduced cell death and lessened oxidant stress by BW373U86 were abolished by 5-HD or the superoxide dismutase inhibitor DDC. Stimulation of δ_1-opioid receptors generates intracellular oxygen radicals by opening mitochondrial K_{ATP} channels. This pathway is important in opioid-produced cardioprotection.

Signal Transduction of Oxygen Radicals

Oxygen radicals activate potassium channels (mediator).[28] K_{ATP} channel activation mediates the cardioprotection of morphine. This protection is abolished by the K_{ATP} channel antagonists 5-HD or glibenclamide.[3] The protection of BW373U86 also was abolished with 5-HD, a selective mitochondrial K_{ATP} channel antagonist. Mitochondrial K_{ATP} channel activation (trigger) increases oxygen radicals,[20,27] which amplifies activation of the channels (mediator) *via* a positive feedback system.

Oxygen radicals (trigger) also activate protein kinase C.[29] Protein kinase C activation mediated opioid protection in intact rabbit hearts,[30] cultured rat ventricular myocytes,[22] and chick embryonic myocytes.[31] Protein kinase C increased the activity of K_{ATP} channels in ventricular myocytes.[31] K_{ATP} channel activation mediates cardioprotection of opioids.[2,3]

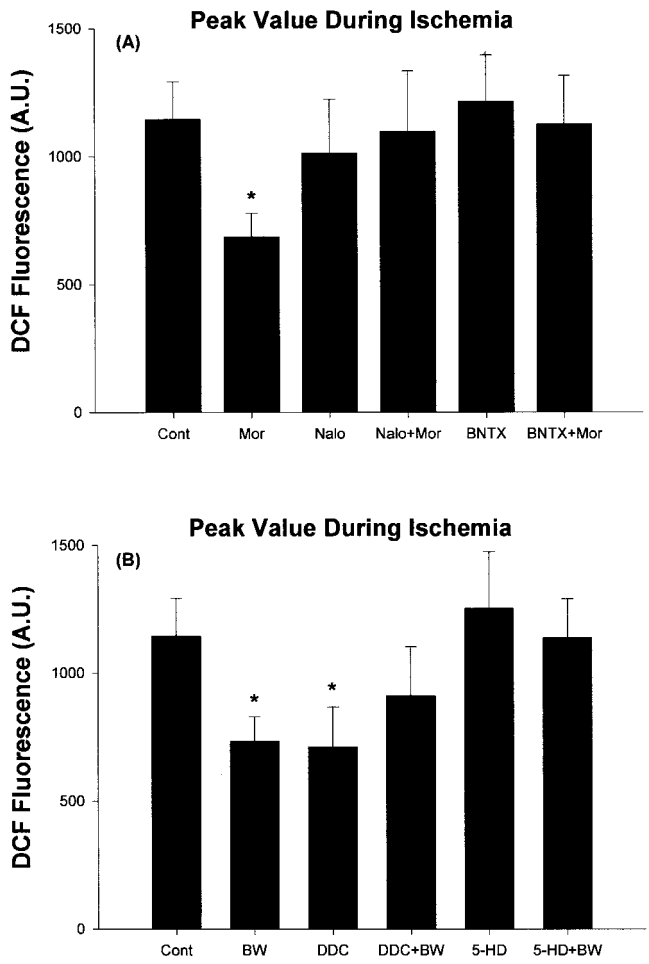

Fig. 4. Oxidant stress during ischemia. Pretreatment with morphine decreased free radical production compared with production in controls (Cont; *A*). Lessened oxidant stress with morphine was restored by pretreatment with naloxone (Nalo+Mor) or 7-benzylidenenaltrexone (BNTX+Mor; *A*). BW373U86 attenuated oxidant stress, which was reversed by diethyldithiocarbamic acid (DDC+BW) or 5-hydroxydecanoate (5-HD+BW; *B*). *$P < 0.05$. DCF = dichlorofluorescein; A.U. = arbitrary units.

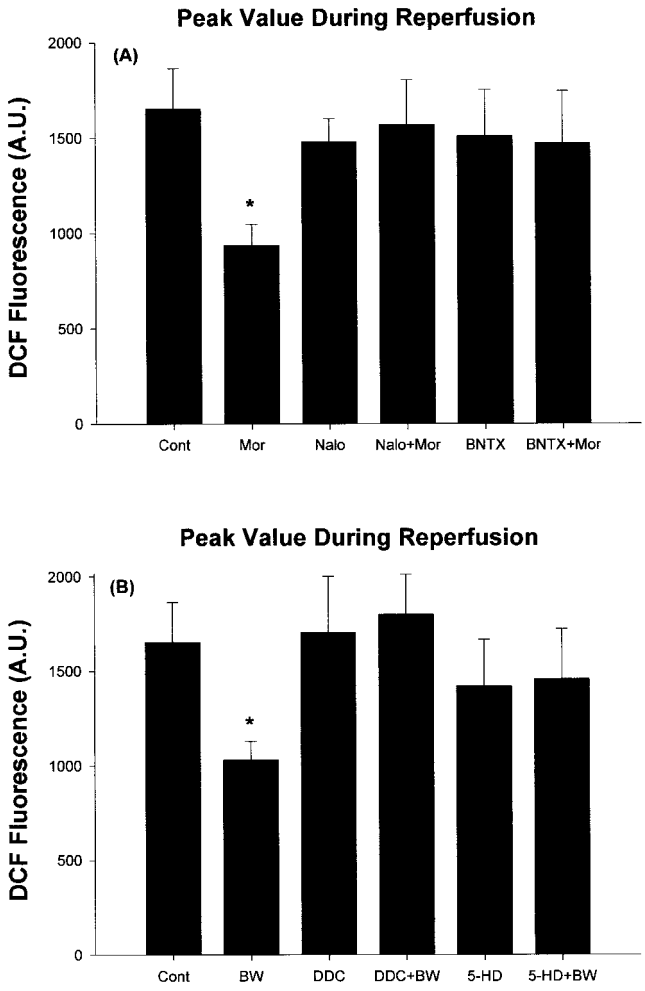

Fig. 5. Oxidant stress during reperfusion. (*A*) Pretreatment with morphine decreased free radical production during reperfusion compared with controls (Cont). Attenuated oxidant stress with morphine was reversed by pretreatment with naloxone (Nalo+Mor) or 7-benzylidenenaltrexone (BNTX+Mor). Attenuated oxidant stress with BW373U86 was abolished by diethyldithiocarbamic acid (DDC+BW) or 5-hydroxydecanoate (5-HD+BW; *B*). *$P < 0.05$. DCF = dichlorofluorescein; A.U. = arbitrary units.

2',7'-Dichlorofluorescin Oxidation and Oxygen Radicals

Although DCFH is widely used to measure oxygen radical generation,[21,32] we recognize the limitation of this assay. The reactive oxygen species that oxidize DCFH to fluorescence-active DCF remains unclear. When superoxide is formed after oxidation to DCF, H_2O_2 is generated in disproportionally small amounts, making the use of the assay to detect it potentially problematic. Changes in peroxidase activity are as important as the changing rate of H_2O_2 formation. Lastly, the nonspecific peroxidase activity of methemoglobin and prostaglandin H synthase is known to oxidize DCFH to DCF.[33]

We allowed experimental cells to equilibrate for at least 40 min to reach a steady state before any interventions. DCFH baseline was monitored during a period of 7 h to ensure no unexpected changes. Our simple system lacks the potential confounding factors present *in vivo,* such as methemoglobin and nonspecific peroxidase activities from other cell types. Finally, any increased DCFH oxidation was abolished by at least two conventional free radical scavengers, including 2-mercaptopropionyl and ebselon. Thus, it is possible but unlikely that our results came from a change in peroxidase activity, autogenerated H_2O_2, or nonspecific enzyme activities in cardiomyocytes.

In conclusion, stimulation of δ_1-opioid receptors generates oxygen radicals (mainly H_2O_2) *via* mitochondrial K_{ATP} channel opening. Through this signaling pathway, opioids attenuate oxidant stress and reduce cell death in cultured cardiomyocytes.

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