Vitamin D receptor activation and downregulation of renin-angiotensin system attenuate morphine-induced T cell apoptosis

Nirupama Chandel, Bipin Sharma, Divya Salhan, Mohammad Husain, Ashwani Malhotra, Shilpa Buch, and Pravin C. Singhal

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Chandel N, Sharma B, Salhan D, Husain M, Malhotra A, Buch S, Singhal PC. Vitamin D receptor activation and downregulation of renin-angiotensin system attenuate morphine-induced T cell apoptosis. Am J Physiol Cell Physiol 303: C607–C615, 2012. First published July 3, 2012; doi:10.1152/ajpcell.00076.2012.—Opiates have been reported to induce T cell loss. We evaluated the role of vitamin D receptor (VDR) and the activation of the renin-angiotensin system (RAS) in morphine-induced T cell loss. Morphine-treated human T cells displayed downregulation of VDR and the activation of the RAS. On the other hand, a VDR agonist (EB1089) enhanced T cell VDR expression both under basal and morphine-stimulated states. Since T cells with silenced VDR displayed the activation of the RAS, whereas activation of the VDR was associated with downregulation of the RAS, it appears that morphine-induced T cell RAS activation was dependent on the VDR status. Morphine enhanced reactive oxygen species (ROS) generation in a dose-dependent manner. Naltrexone (an opiate receptor antagonist) inhibited morphine-induced ROS generation and thus, suggested the role of opiate receptors in T cell ROS generation. The activation of VDR as well as blockade of ANG II (by losartan, an AT1 receptor blocker) also inhibited morphine-induced T cell ROS generation. Morphine not only induced double-strand breaks (DSBs) in T cells but also attenuated DNA repair response, whereas activation of VDR not only inhibited morphine-induced DSBs but also enhanced DNA repair. Morphine promoted T cell apoptosis; however, this effect of morphine was inhibited by blockade of opiate receptors, activation of the VDR, and blockade of the RAS. These findings indicate that morphine-induced T cell apoptosis is mediated through ROS generation in response to morphine-induced downregulation of VDR and associated activation of the RAS.

Opiate addicts are prone to infections (5, 12, 24, 33). Partly, it has been attributed to opiate-induced loss of T cells (30, 31). Severity of CD4+ve T cell depletion has been demonstrated to be a major determinant for occurrence of life-threatening bacterial infections in patients with AIDS (2). Since both HIV and morphine have been demonstrated to induce T cell apoptosis (2, 35), opiate addicts with HIV infection are more vulnerable to incur T cell loss.

Naïve human T cells have been reported to display very low expression of vitamin D receptor (VDR) (32). However, primed T cells exhibited robust VDR expression via T cell receptor-activated induction of the alternative p38 pathway (32). The role of p38 and VDR induction has also been demonstrated in several other cells (22, 23). In these studies, p38 mediated the activation of transcription factors such as AP-1 and ATF2, which interacted with their corresponding elements in the promoter of the gene encoding VDR (22, 23). VDR mediates most (growth arrest, apoptosis, and differentiation) if not all of the biologic effects of 1,25(OH)2D3 (active product of vitamin D) (7, 34). Most analogs of 1,25-dihydroxy vitamin D2 or D3 act via the VDR, although some have effects unrelated to VDR binding such as CYP24 enzyme inhibition (7, 32). 1,25(OH)2D3 stabilizes the VDR and inhibits its degradation (8). VDR shuttles constantly between the nucleus and cytoplasm and has been shown to be a negative regulator of renin transcription and NF-κB signaling (20, 21). Both renin and NF-κB have been demonstrated to contribute to the production of ANG II, the most active molecule of the renin-angiotensin system (18, 28).

The activation of T cell renin-angiotensin system (RAS) and associated generation of reactive oxygen species (ROS) have been demonstrated to contribute to progressive kidney cell injury in patients with hypertension (19). Although oxidative stress has been demonstrated to be one of the key factors contributing to T cell apoptosis, the role of RAS in morphine-induced ROS generation in T cell apoptosis has not been studied to date. We hypothesized that morphine-induced T cell RAS activation contributes to morphine-induced ROS generation and associated apoptosis.

In the present study, we have not only delineated the mechanisms involved in T cell loss in a morphine milieu but we have also tested strategies to prevent their loss. Morphine-induced downregulation of VDR led to the activation of the RAS, generation of ROS, DNA damage, and apoptosis of T cells. Conversely, a VDR agonist restored T cell expression of VDR, downregulated the RAS, attenuated ROS generation and DNA damage, and thus, sustained the survival phenotype. Similarly, both inhibition of opiate receptors and blockade of the RAS provided protection against morphine-induced T cell injury. Thus, the present study not only delineates the molecular mechanisms involved in morphine-induced T cell loss but also helps to develop therapeutic strategies to prevent T cell loss in a morphine milieu.1

METHODS AND MATERIALS

Human T cells. T cells were isolated from blood samples obtained from healthy volunteers (New York Blood Bank, New York). Peripheral blood mononuclear cells (PBMCs) were harvested by the standard technique. T cells were isolated from PBMCs by passing through the T cell column (Invitrogen, Oslo, Norway). T cells were primed before their use (IL-2, 20 U/ml and phytohemagglutinin P, 5.0 μg/ml).

1 This article is the topic of an Editorial Focus by Ulrich Hopfer (15a).
Since Jurkat cells (human T cell line, with intact T cell receptor, National Institutes of Health AIDS Research and Reference Reagent Program, Bethesda, MD) are comparable to primed T cells and they constitutively express phospholipase C-γ1 (17), we have also used them in place of primary primed human T cells.

**TUNEL assay.** TUNEL assay was performed using Apoptosis Detection Tacs TdT Kit (R&D Systems, Minneapolis, MN) (16). Briefly, the cells were fixed in 3.7% formaldehyde in 1 × phosphate-buffered saline (PBS). The cells were permeabilized in Cytomin solution for 15 min at room temperature and then quenched in 3% H2O2 in methanol for 5 min. The cells were washed and treated with TdT labeling buffer followed by another PBS wash. The cells were incubated in streptavidin-horseradish peroxidase solution for 10 min followed by washing in PBS and incubation in diaminobenzidine substrate solution for 10 min. The cells were rinsed in water and counterstained with Nuclear Fast Red. The coverslips were mounted on the slide using aqueous mounting media and observed under light microscope.

**Immunofluorescence detection of oxidant stress in T cells.** The trafficking of 2,3,4, and 5,6-pentafluorodihydrotetramethylrosamine (PF-HTMRos or Redox Sensor Red CC-1, Molecular Probes, Eugene, OR) was used to detect reactive oxygen intermediates in T cells (16, 27). Redox Sensor Red CC-1 is oxidized in the presence of O2 and H2O2. In brief, control and experimental T cells were loaded at 37°C for 20 min with Redox Sensor Red CC-1 (0.5 μM) and a mitochondria-specific dye, MitoTracker green FM (100 nM; Molecular Probes). Culture slides were washed and mounted with PBS and visualized with Nikon fluorescence microscope (Nikon Eclipse E800) equipped with triple filter cube and charge-coupled device (CCD) camera (Nikon DXM1200). The staining was performed in quadruplicate for each group, and 10 random fields were studied in replicate. Images were captured using Nikon ACT-1 (version 1.12) software and combined for publishing format using Adobe Photoshop 6.0 software.

**Immunofluorescence detection of DNA strand breaks and repairs.** Control and experimental T cells were fixed and permeabilized with a buffer containing 0.02% Triton X-100 and 4% formaldehyde in PBS. Fixed cells were washed three times in PBS and blocked in 1% BSA for 30 min at 37°C. Phospho-histone H2AX (γH2AX) was detected by a mouse monoclonal antibody that recognizes phosphorylated serine within the amino acid sequence 134–142 of human histone H2A.X (UBI) and FITC-conjugated goat anti-mouse secondary antibody (Molecular Probes). KU80, a DNA repair protein, was detected by rhodamine-conjugated anti-KU80 antibody (Cell Signaling, Danvers, MA). Double labeling was indicated by orange color. Negative controls were performed in the presence of nonspecific isotype antibodies in place of primary antibody. In all variables, DNA was counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Specific staining was visualized with an inverted Olympus 1X 70 fluorescence microscopes equipped with a Cook Sensicam ER camera (Olympus America, Melville, NY). Final images were prepared with Adobe Photoshop to demonstrate subcellular localization of γH2AX (27).

**Determination of ROS kinetics in T cells.** The kinetics of ROS metabolism in T cells was determined by measuring the intensity of the fluorescent signal from the redox-sensitive fluoroprobe 2,7′-dichlorofluorescein diacetate (DCFDA) at multiple time points. DCFDA is converted by intracellular esterases to 2,7′- dichlorodihydrofluorescein, which in turn is oxidized by H2O2 to the fluorescent 2′,7′-dichlorodihydrofluorescein (DCF). Briefly, control and experimental T cells were incubated in phenol red-free media containing 10 mM DCFDA for 30 min at 37°C. Cells were washed with phenol red-free media, and DCF fluorescence was detected by a Fluorescence Multi-Well Plate Reader CytoFluor 4000 (PerSeptive Biosystems) set for excitation of 485 nm and emission of 530 nm. The intensity of the fluorescent signal was calculated with Microsoft Excel using equation \([\frac{[F_t - F_0]}{F_0} \times 100]\) (16, 27).

**Silencing of VDR.** Jurkat cells were transfected with 100 nM VDR small interfering (si)RNA (Santa Cruz Biotechnology, Santa Cruz, CA) with Siport Neoax transcription reagent and left in optiMEM media for 24 h. Control and transfected cells were used under control and experimental conditions.

**ANG II ELISA.** ANG II levels were determined in control and experimental T cells using commercial ELISA kits (Peninsula Laboratories, Belmont, CA) as described by the manufacturer. Briefly, ANG II was extracted with 20 mM Tris buffer, pH 7.4, and partially purified and concentrated after filtration through Centricon Filters (MW cutoff 10,000, Millipore, Billerica, MA).

**Real-time PCR analysis.** Control and experimental cells were used to quantify mRNA expression of molecules pertaining to VDR, renin, and angiotensinogen (Agt). RNA was extracted using TRIzol (Invitrogen). For cDNA synthesis, 2 μg of the total RNA was preincubated with 2 nmol of random hexamer (Invitrogen) at 65°C for 5 min. Subsequently, 8 μl of the reverse transcription (RT) reaction mixture containing cloned AMV RT, 0.5 nmol each of the mixed nucleotides, 0.01 mol dithiothreitol, and 1,000 U/ml Rnasin (Invitrogen) was incubated at 42°C for 50 min. For a negative control, a reaction mixture without RNA or RT was used. Samples were subsequently incubated at 85°C for 5 min to inactivate the RT.

Quantitative PCR was carried out in an ABI Prism 7900HT sequence detection system using the primer sequences as shown in Table 1.

**SYBR green was used as the detector and ROX as a stabilizing dye. Results (means ± SD) represent three to four sets of experiments as described in the figure legends. The data were analyzed using the comparative Ct method (ΔΔCt). Differences in Ct are used to quantify the relative amount of PCR target contained within each well. The data are expressed as relative mRNA expression in reference to control and were normalized to the quantity of RNA input by performing measurements on an endogenous reference gene, GAPDH.**

**Western blot studies.** T cells were lysed in RIPA buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% deoxycholate, 0.1% SDS, 1× protease inhibitor cocktail I (Calbiochem, EMD Biosciences, Gibbstown, NJ), 1 mM PMSF, and 0.2 mM sodium orthovandate. Protein concentration was measured with the Bio-Rad Protein Assay kit (Pierce, Rockford, IL). Protein lysates (20 μg) were separated on a 15% polyacrylamide gels (PAGE, Bio-Rad, Hercules, CA) and transferred onto a nitrocellulose membrane using a Bio-Rad miniblot apparatus. Nitrocellulose membranes were then subjected to immunostaining with primary antibodies against VDR (mouse monoclonal, Santa Cruz), Agt (rabbit polyclonal, Epitomics), renin (rabbit polyclonal, Santa Cruz), and subsequently

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**Table 1. Primer sequences used for quantitative PCR**

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<th>Gene (Gene Bank No.)</th>
<th>Primer Sequence (5′-3′)</th>
<th>Annealing Temperature</th>
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<tr>
<td>Renin (L00070 to L00073)</td>
<td>5′-GGCGGATATTTCCATGAGGAGGTTCGTC-3′; 5′-AGGGGAGGCAAATCCGCTTGATTC-3′</td>
<td>58°C</td>
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<tr>
<td>AGT (X15325, X15326)</td>
<td>5′-CTGGCGCAGCGCGCAGTTGTCG-3′; 5′-TCAAGTGTGAGTTGTCGGCGGAGAC-3′</td>
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<tr>
<td>VDR (NM_000376.2)</td>
<td>5′-GACTTGGAGCGGAGACCTGCG-3′; 5′-CATCACTGCAGCGAGTGACCAAGCA-3′</td>
<td>58°C</td>
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AGT, angiotensinogen; VDR, vitamin D receptor.
with horseradish peroxidase-labeled appropriated secondary antibodies. The blots were developed using a chemiluminescence detection kit (Pierce) and exposed to X-ray film (Eastman Kodak, Rochester, NY). Equal protein loading was confirmed by stripping the blot and reprobing it for actin protein using a polyclonal α-actin antibody (Santa Cruz) on the same Western blots.

Statistical analysis. For comparison of mean values between two groups, the unpaired t-test was used. To compare values between multiple groups, analysis of variance was used to calculate a P value. Statistical significance was defined as P < 0.05. Results are presented as means ± SD.

RESULTS

Morphine downregulates T cell VDR. To determine the effect of morphine on T cell VDR, we evaluated the dose-response effect of morphine on T cell VDR expression. As shown in Fig. 1A, morphine downregulated VDR expression in a dose-dependent manner.

To evaluate the time course effect of morphine on VDR, we determined the effect of morphine on T cell VDR expression at different time periods. Morphine diminished T cell VDR expression at all time periods (Fig. 1B).

To determine the effect of morphine on Jurkat cell VDR expression, Jurkat cells were treated by morphine for 24 h. Morphine downregulated Jurkat cell VDR expression (Fig. 1C). Results are shown in a bar diagram.

To determine the effect of ANG II blockade on the effect of morphine-induced downregulated T cell VDR expression, Jurkat cells were incubated in media containing buffer, morphine, or morphine + losartan for 24 h. Protein blots probed for VDR; the same blots were reprobed for actin. As shown in Fig. 1D, losartan did not alter the effect of morphine.

Morphine upregulates RAS. To determine the effect of morphine on T cell RAS status, T cells were incubated in media containing either buffer or morphine. Protein blots were probed for renin and actin. Gels from three different cellular lysates as well as densitometric data are shown in Fig. 2A. Morphine displayed twofold increase (P < 0.01) in T cell renin expression compared with control.

Since Agt is the substrate for renin, we evaluated the effect of morphine on T cell Agt expression. Protein blots prepared from control and morphine-treated T cells were probed for Agt and actin. Gels from three different cellular lysates as well as densitometric data are shown in Fig. 2B. Morphine enhanced (P < 0.01) T cell Agt expression by twofold compared with control.

To determine the effect of morphine on T cell ANG II production, T cells treated under control and morphine-treated conditions were assayed for their ANG II levels. Results are shown in Fig. 2C. Morphine stimulated (P < 0.01) T cell ANG II production by fivefold.

Lack of VDR is essential for T cell production of ANG II. To establish cause and effect relationship between lack of VDR and T cell ANG II production, cellular lysates of control T cells, T cells transfected with siRNA-VDR, and T cells trans-
Jurkat cells were incubated in media containing either buffer or morphine (10^{-6} M) for 24 h. Proteins from three different cellular lysates were probed for renin. The same blots were reprobed for actin. The top lane displays renin expression by control and morphine-treated Jurkat cells. The bottom lane shows Jurkat cell actin expression under similar conditions. Results (means ± SD) are shown in a bar diagram. B: Jurkat cells were incubated in media containing either buffer or morphine (10^{-6} M) for 24 h. Cellular lysates from three different experiments were electrophoresed and probed for angiotensinogen (Agt). The same blots were reprobed for actin. The top lane shows Agt expression by control and morphine-treated Jurkat cells. The bottom lane displays Jurkat cell actin expression under the same conditions. Results (means ± SD) are shown in bar graphs. C: Jurkat cells were incubated in media containing either buffer or morphine (10^{-6} M) for 24 h. At the end of the incubation, cellular lysates were assayed for ANG II. Results (means ± SD) represent four sets of experiments. D: Jurkat cells were transfected with small interfering (si)RNA-VDR or scrambled (SCR) siRNA. Representative gels of control, siRNA-VDR/Jurkat, and SCR-siRNA/Jurkat are shown. ANG II content was measured in control, SCR-siRNA-transfected, and VDR-siRNA-transfected Jurkat cells. Results (means ± SD) represent four sets of experiments. *P < 0.001 compared with other variables.

**Role of opiate receptor in morphine-induced T cell renin expression.** To evaluate the role of opiate receptors, we determined the effect of naltrexone (an opiate receptor antagonist) on morphine-induced T cell renin expression. Protein blots of control, morphine-, naltrexone-, or morphine + naltrexone-treated Jurkat cells were probed for renin and actin. Morphine upregulated Jurkat cell renin expression (Fig. 4A); however, this effect of morphine was attenuated by naltrexone.

**Naltrexone and VDA inhibit morphine-induced T cell VDR downregulation and the activation of the RAS.** To determine the effect of naltrexone and VDA on morphine-modulated transcription of Jurkat cell VDR expression, Jurkat cells were pretreated with either naltrexone (10^{-5} M) or VDA (10 nM) and then treated with either buffer or morphine (10^{-6} M) for 24 h. Subsequently, total RNA was extracted. mRNA expression of VDR, renin, and Agt was quantified by real-time PCR studies. Morphine attenuated Jurkat cell mRNA expression for VDR (Fig. 4B); however, both naltrexone and VDA inhibited this effect of morphine. Although morphine attenuated Jurkat cell VDR expression under basal state, it enhanced both renin (Fig. 4C) and Agt (Fig. 4D) mRNA expression by Jurkat cells. However, both naltrexone and VDA inhibited this effect of morphine.

**Morphine stimulates T cell ROS generation.** To determine the effect of morphine on T cell ROS generation, Jurkat cells were loaded with DCFDA, followed by treatment with morphine (10^{-10} M to 10^{-6} M) and measurement of ROS generation.
To establish a causal relationship between morphine/VDR/RAS and induction of T cell apoptosis. Jurkat cells pretreated with VDA and losartan were incubated in media containing either buffer or morphine. Subsequently, cells were colabeled with Red CC1 and MitoTracker Green. Representative microphotographs are shown in Fig. 6C. Morphine-treated Jurkat cells displayed mitochondrial generation of ROS. Both VDA and losartan attenuated morphine-induced ROS generation.

Effect of VDA activation, ANG II blockade, and antioxidants in morphine-induced T cell double-strand breaks and DNA repair. To determine the effect of VDA, losartan, catalase (free radical scavenger) and Tempol (dismutase mimetic) on morphine-induced DNA damage, control, morphine-, or morphine + VDA-, morphine + catalase-, and morphine + Tempol-treated Jurkat cells were colabeled for H2AX (double-strand breaks) and KU80 (H2AX, DNA repair protein) expression. Morphine enhanced double-strand breaks in Jurkat cells and displayed only moderate expression of KU80 in morphine-treated cells. These findings indicate that downregulation of VDR was associated with inadequate DNA repair response, whereas the activation of VDR, blockade of ANG II, and scavenging of ROS were associated with diminished double-strand breaks and enhanced DNA repair response. Figure 7, B and C, summarizes the data as scattergrams, with horizontal lines indicating means.

Establishment of causal relationship between morphine/VDR/RAS and induction of T cell apoptosis. To establish a causal relationship between morphine/VDR/RAS and the induction of morphine-induced T cell apoptosis, Jurkat cells were pretreated with naltrexone, VDA, and losartan, followed by treatment with either buffer or morphine. Subsequently,

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<th>A</th>
<th>VDR</th>
<th>Actin</th>
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<td>Control</td>
<td>Morphine</td>
<td>Morphine/VDA</td>
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<tr>
<th>B</th>
<th>ANG II pg/mg protein</th>
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Fig. 3. Vitamin D stimulates T cell VDR expression and attenuation of ANG II production. A: Jurkat cells were incubated in media containing either buffer, morphine (10^{-6} M), VDR agonist (VDA; 10 nM), or morphine + VDA for 24 h. Cellular lysates of two different experiments were electrophoresed and probed for VDR and actin. The top lane displays Jurkat cells VDR expression under indicated conditions. The bottom lane shows Jurkat cell actin expression under the same conditions. B: Jurkat cells were incubated in media containing either buffer, morphine (10^{-6} M), VDA (10 nM), or morphine + VDA for 24 h. Cellular lysates were assayed for ANG II by ELISA. Results (means ± SD) represent four sets of experiments. *P < 0.001 compared with control; **P < 0.01 compared with morphine.

Fig. 4. Naltrexone and VDA inhibit morphine-induced T cell ROS generation and the activation of the RAS. A: Jurkat cells were incubated in media containing buffer, morphine (M, 10^{-7} M), naltrexone (Nal, 10^{-6} M), or morphine + naltrexone for 24 h. Western blots from two different cellular lysates were probed for renin; the same blots were reprobed for actin. The top lane displays Jurkat cells renin expression under control and experimental conditions. The bottom lane shows Jurkat cell actin expression under the same conditions. B: Jurkat cells were pretreated with either buffer, VDA (10 nM), or naltrexone (10^{-6} M) for 6 h followed by incubation in media containing either buffer or morphine (10^{-6} M). At the end of the incubation period, total RNA was extracted and probed for VDR by real-time PCR. Results (means ± SD) represent three different sets of experiments. *P < 0.001 compared with control (C), morphine + naltrexone, and morphine + VDA; **P < 0.01 compared with control. C: total RNA was extracted from Jurkat cells treated similarly to B. mRNA expression for renin was assayed by real-time PCR. Results (means ± SD) represent three different sets of experiments. *P < 0.001 compared with control; **P < 0.01 compared with morphine. D: total RNA was extracted from Jurkat cells treated similarly to B. mRNA expression for Agt was assayed by real-time PCR. Results (means ± SD) represent three different sets of experiments. *P < 0.001 compared with control; **P < 0.01 compared with morphine.
cells were assayed for apoptosis both by TUNEL assay and FACS analysis. Representative microphotographs and cumulative data of control, morphine-, morphine + naltrexone-, morphine + VDA-, and morphine + losartan-treated Jurkat cells are shown in Fig. 8. Morphine promoted \((P < 0.001)\) T cell apoptosis; however, this effect of morphine was partially inhibited \((P < 0.01)\) by naltrexone, VDA, and losartan (Fig. 8). Occurrence of apoptosis in T cells treated with naltrexone alone, VDA alone, or losartan alone was comparable to control cells (data not shown).

To determine the effect of antioxidant on morphine-induced apoptosis, Jurkat cells were pretreated with either buffer or Tempol then treated with either buffer or morphine for 24 h. Subsequently, cells were assayed for apoptosis by TUNEL assay. As shown in Fig. 9, morphine enhanced \((P < 0.01)\) T cell apoptosis; however, this effect was inhibited by Tempol.

**DISCUSSION**

In the present study, morphine downregulated VDR expression in primed primary human T cells and Jurkat cells. Morphine-induced T cell VDR downregulation was associated with the activation of the RAS. VDA enhanced T cell VDR expression both under basal and morphine-stimulated states. VDA also attenuated morphine-induced ANG II production by T cells. Morphine enhanced ROS generation in a dose-dependent manner. Since naltrexone inhibited morphine-induced ROS generation as well as apoptosis, it suggests that morphine-
induced T cell ROS generation and apoptosis were mediated through opiate receptors. Both the activation of VDR and blockade of ANG II also inhibited morphine-induced T cell ROS generation and apoptosis. Morphine not only induced double-strand breaks but also compromised DNA repair response in T cells, whereas activation of VDR not only diminished morphine-induced double-strand breaks but also enhanced DNA repair in morphine-treated T cells. These findings indicate that morphine-induced T cell apoptosis is mediated through ROS generation in response to morphine-induced downregulation of VDR and associated activation of the RAS.

VDR has been reported to be a negative regulator of renin (20, 21). In the present study, morphine not only downregulated T cell VDR expression but also enhanced renin expres-

Fig. 7. Effect of VDR activation, ANG II blockade, and antioxidants in morphine-induced T cell double-strand breaks and DNA repair. A: Jurkat cells were incubated in serum-free media containing either buffer or morphine (M, 10⁻⁶ M) in the presence or absence of VDA (10⁻⁷ M), losartan (10⁻⁷ M), catalase (CAT, 500 units), or Tempol (TEMP, 1 μM) for 24 h. Subsequently, cells were colabeled with anti-H2AX and anti-KU80 antibodies along with nuclear stain (DAPI) (n = 3). Representative microphotographs are shown. Control cells displayed baseline double-strand breaks (green fluorescence), whereas morphine (M)-treated cells showed enhanced number of double-strand breaks; however, VDA, LOS, CAT, and TEMP attenuated morphine-induced double-strand breaks. Similarly, morphine attenuated expression of KU80 compared with control, whereas M/VDA, M/LOS, M/CAT, and M/TEMP cells displayed increased KU80 expression compared with morphine alone. B: cumulative data displaying number of H2AX foci from the experimental protocol described in A are shown in the form of a scattergram. C: cumulative data displaying integrated density of KU80 from the experimental protocol described in A are shown in the form of a scattergram.

Fig. 8. Establishment of causal relationship between morphine/VDR/RAS and induction of T cell apoptosis. Jurkat cells were pretreated with naltrexone (10⁻⁵ M), VDA (10 nM), and losartan (10⁻⁷ M), followed by incubation in media containing either buffer or morphine (10⁻⁶ M) for 24 h. Subsequently, cells were assayed for apoptosis by TUNEL assay. Representative microphotographs are shown. TUNEL +ve cells are stained blue (indicated by arrows). Cumulative data of the indicated conditions are shown in bar graphs. *P < 0.001 compared with control; **P < 0.01 compared with morphine.
sion; on the other hand, VDA reversed these effects of morphine. Interestingly, losartan also inhibited morphine-induced ROS generation as well as apoptosis. These findings indicate that the morphine-induced RAS activation contributed to T cell loss. Since T cells with silenced VDR displayed enhanced production of ANG II, whereas activation of VDR diminished morphine-induced T cell ANG II production, it confirms that VDR acted as a negative regulator of the RAS in T cells.

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen and are highly reactive due to the presence of unpaired valence shell electrons, which can be damaging if their generation is not limited (29). ROS are generated as by-products of the incomplete four-electron reduction of molecular oxygen to water (25, 29). The electron transport chain of mitochondria is the major source of intracellular ROS in the cell and mitochondria are also a major target for damage by ROS (3, 4). In the present study, morphine also stimulated T cell ROS generation predominantly in mitochondria. It will be important to study the effect of morphine on T cell mitochondrial damage in future studies.

Oxidative stress has been reported to be a key mediator of apoptosis in lymphoid cells (6). Lymphocytes are under continuous surveillance—whether they should be preserved or programmed to die (6). ROS targets polyunsaturated fatty acids, which are abundant in replicating lymphocytes (15). Usually, an organism’s capacity to repair oxidative DNA damage determines cellular survival (9–11). The two major repair mechanisms for double-strand breaks (DSBs) are homologous recombination (HRR) and nonhomologous end joining (NHEJ) (11). Oxidative DNA damage also activates the p53 pathway to target DNA repair genes (13, 14). In the present study, morphine-treated cells displayed enhanced ROS generation and increased DSBs but displayed diminished expression of KU80, a DNA repair protein. These findings suggest that morphine-induced DNA injury is accompanied by a compromised DNA repair response. The net outcome of these two events has contributed to morphine-mediated untoward outcome, T cell apoptosis.

In the present study, we have used morphine concentrations ranging from $10^{-10}$ M to $10^{-6}$ M. These concentrations are relevant to physiological levels observed in opiate addicts (1, 26). Similarly, we have used E1089 in nanomolar concentrations which are also physiological and have been used by other investigators (20, 21).

Figure 10 shows a diagram of our proposed mechanism in morphine-induced apoptosis and therapeutic strategies to prevent it. In brief, morphine enhances the activation of the RAS by downregulation of T cell VDR expression. The RAS stimulates ROS generation by T cells and associated DNA damage and T cell apoptosis. The latter can be prevented either by inhibition of opiate receptors (naltrexone), VDR agonists, ANG II blockers, and free radical scavengers.

The present study not only delineates the molecular mechanism involved in morphine-induced T cell loss but also provides opportunities to develop therapeutic strategies to provide protection against T cell loss in a morphine milieu.

ACKNOWLEDGMENTS

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

N.C. and D.S. performed the experiments; N.C., B.S., D.S., M.H., A.M., S.B., and P.C.S. approved the final version of the manuscript; B.S. prepared the figures; M.H. analyzed the data; A.M. and S.B. interpreted the results of the experiments; P.C.S. conception and design of the research; P.C.S. edited and revised the manuscript.
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