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Effect of Analgesic Opioid Drugs on Opioid Receptor Genes Expression in HL-1 Mouse Cardiac Myocytes

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Abstract

BACKGROUND: The opioid system was mainly involved three types of opioid receptors (ORs): μ (MOR), δ (DOR) and κ (KOR). These ORs are activated by its agonist, a family of endogenous peptides: Endorphins, enkephalins, and dynorphins, respectively.

AIM: This study determined the OR mRNA on effects of agonists exogenous morphine, fentanyl, D-penicillamine (2,5) enkephalin, and ketazocine in HL-1 mouse cardiac myocytes.

MATERIALS AND METHODS: HL-1 mouse cardiac myocytes were treated with 10 μM morphine sulfate, 1 μM fentanyl,1 μM D-penicillamine (2,5) enkephalin, and 1 μM ketazocine. Total mRNAs were extracted and cDNA was synthesized and quantitative real-time polymerase chain reaction was used to analyze gene expression.

RESULTS: The data analysis of MOR, DOR and KOR mRNA expression on effect of morphine was shown less level than control (0.61-fold, 0.67-fold, and 0.65-fold), respectively. The morphine-induced ORs down-regulation, whereas enkephalin treatment demonstrated highly significantly increased in mRNA of DOR (6.3-fold, p = 0.002). As well as, KOR mRNA expression was found highly significant increased under effect of Ketazocine (7.16-fold, p = 0.004).

CONCLUSION: This study found DOR and KOR, but not MOR expressed in HL-1 mouse cardiac myocytes under activation of exogenous opioid analogists. These findings suggested that exogenous analogist's opioids mimeses the endogenous analogist's opioids.

Introduction

Regarding to pharmacological and molecular biological studies the opioid system was mainly classified into three types of opioid receptors (ORs): mu (MOR), delta d (DOR), and kappa k (KOR) [1]. Recently, a novel type of "opioid receptor-like orphan receptor" (ORL-1) was discovered for 20 years ago, also known as the nociception receptor [2]. All four are members of the seven transmembrane spanning G-protein coupled receptor family [3]. The three ORs are encoded by three different genes with highly conserved in their regulatory sequences, but expressed in spatially and temporally various patterns. Studies using models of different cells have shown combinatorial transcriptional regulation through multiple transcription factors [4]. Mu (μ), delta (δ), and kappa (κ) ORs are activated by its agonist, a family of endogenous peptides: Endorphins, enkephalins, and dynorphins, respectively, whereas nociceptin/orphanin FQ is ORL-1 natural ligand of the ORL-1 receptor. In addition, Mu (μ), delta (δ), kappa (κ) ORs are also activated by exogenous agonists: Morphine, D-penicillamine (2,5) enkephalin, and ketazocine, respectively [5]. While ORL-1 may play a role in the development of tolerance to μ agonists, in spite of this effect has not been confirmed yet. Despite of ischemic heart disease and resultant acute myocardial infarction is still one of the most reasons of mortality globally, the capability to effectively interfere and minimize myocardial damage during ischaemia–reperfusion (Clinical cardioprotection) stays a largely unachieved therapeutic target. A range of protective process have been reported as highly efficacious in lab experiments, with particular focus on ischemic pre- and post-conditioning and related stimuli [6], [7]. However, previous studies have reported that motivation of both cardiac and central ORs is able to evoke heart function and trigger cardioprotection against ischemia [8], [9]. The effectiveness of opioids in inducing pre- and post-conditioning in hearts *in vivo* or *in vitro* can be achieved [10]. Gwag *et al*. study has been demonstrated that morphine resultant cardioprotective through activation of ORs and could be reduced myocardial infarct size and reperfusion injury in similar way to that of ischemic preconditioning [11]. As well as, previous laboratory studies on experimental animals have confirmed this finding [12]. Similar cardioprotective effects have been found for other ORs agonists such as fentanyl and remifentanil [13]. Therefore, our study was aimed to investigate the expression of OR genes upon effects of exogenous Morphine, fentanyl, D-penicillamine (2,5) enkephalin, and ketazocine on cardiomyocytes.

Materials and Methods

Cell culture

HL-1 mouse cardiac myocytes (Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution. The cells were incubated in 95% air and 5% CO_2 at 37°C. Culture medium was replaced every 2–3 days, and cells were sub cultured at 80% confluence. Following 24 h serum starvation (DMEM supplemented with 1% FBS) before the experiments.

Drugs and treatments

All substance was dissolved in deionized water and HL-1 cardiac myocytes were exposed to 4 treatment schedules beside to control. Control cells received an equal volume of the vehicle (0.1%). The first cells were treated with 10 μM morphine sulfate (Gr1). The second cells were treated with 1 μM fentanyl (Gr2). The third cells were treated with 1 μM D-penicillamine (2,5) enkephalin (Gr3). The fourth cells were treated with 1 μM ketazocine (Gr4). All culture plates were incubated for 24 h continuously without further renewal of growth medium in the incubator. All substances were obtained from Sigma-Aldrich® (St. Louis, MO, USA). At the end of 24 h treatment, cells were washed 3 times with phosphate-buffered saline (PBS) to remove the drug compounds and the serum proteins.

mRNA extraction

At the end of 24 h treatment, all treated cells were washed three times with PBS to remove the drug compounds and the serum proteins. Then the cells were harvested using cell scrapers, and centrifuged at 1500 rpm for 3 min. The cell-pellets were re-suspended in 1 mL PBS, and transferred into Eppendorf tubes, and subjected to centrifugation at 1000 rpm for 3 min. Finally, the pellets were homogenized in 1 mL TRIzol reagent with VirTishear polytron homogenizer (Virtis Company, Inc., Cardiner, NY, USA). Total mRNAs were extracted and purified by using MagNA high Pure system (Roche Diagnostics; Mannheim, Germany), according to manufacturer's protocol. The extracted mRNA was quantified using Nano-drop System (Thermo Scientific, USA).

Reverse transcription (cDNA synthesis)

Single-stranded cDNA was synthesized using 1.0 μg total RNA and the High-Capacity cDNA Reverse Transcription Kit (NEB, USA). First strand cDNA synthesis was performed using a "First strand cDNA synthesis kit with a 24 T primer (0, 4 nmoL per reaction) in a 25 μL reaction mix according to the manufacturer's protocol. The cDNA products were diluted 10-fold before run in real-time polymerase chain reaction (RT-PCR).

Quantitative RT-PCR

The relative levels of MOR, DOR, and KOR mRNA were presented as the normalized ratio of target genes/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (percent). The sequences of gene-specific primers of MOR, DOR, and KOR for RT-PCR are shown in Table 1 [14]. All samples were run in triplicate and all data were normalized to GAPDH as the endogenous reference gene. Quantitative RT-PCR (qRT-PCR) was done in a Roche light cycler, with the use of SYBR Green light mix (Roche, Germany), with 10 μL SYBR Green mix, 2 μL of each primer (6 μM and 1 μL of cDNA in a total volume of 20 μL). Reactions were conducted under the following conditions: 95°C for 3 min, followed by 40 cycles at 95°C for 10 s, 54°C for 30 s, and 72°C for 30 cycles. The different samples were amplified in the same plate with the standard under identical conditions. At the end of each run a melting curve analysis was done (from 55°C to 95°C) to determine the formation of specific products. Relative expression of different ORs gene transcripts were calculated by the $\Delta\Delta C_T$ method and converted to relative expression ratio $2^{-\Delta\Delta CT}$ for statistical analysis [15].

Table 1: Primer sequences of opioid receptors used in AQ-real-time real time-polymerase chain reaction

Primers' Names	Primer Sequences
m (MOR) primers	Forward 5'- TACCGTGTGCTATGGACTGAT-3'
	Reverse 5'- ATGATGACGTAAATGTGAATG-3'
	Probe 5'- CTTGCGCCTCAAGAGTGTCCGCA-3'
d (DOR) primers	Forward 5'-GCGGGAAAGCCAGTGACTC-3'
	Reverse 5'- TGCCCTGTTTAAGGACTCAGTTG-3'
	Probe 5'- AGGAGAGGAGCGGGACCTGTGGCT-3'
k (KOR) primers	Forward5'- CGTCTGCTACACCCTGATGATC-3'
	Reverse5'- CTCTCGGGAGCCAGAAAGG-3'
	Probe 5'- TGCGTCTCAAGAGCGTCCGGC-3'
GAPDH primers	Forward 5'- GGAAGCTCACTGGCATGGC-3'
	Reverse 5'- TAGACGGCAGGTCAGGTCCA-3'
	Probe 5'- CCCCACTGCCAACGTGTCAGTG-3'

MOR: Mu-opioid receptor, DOR: Delta-opioid receptor, KOR: Kappa-opioid receptor, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, PCR: Polymerase chain reaction.

Statistical analysis

Calculated relative OR mRNA levels in different treatment groups were compared using a one-way ANOVA followed by Newman–Keuls test with GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). Differences were considered significant if $p \leq 0.05$.

Results

To evaluate the effect of specific opioid drugs on the expression levels of MOR, DOR, and KOR genes in HL-1 cardiac myocytes using q-RT-PCR. Gene

expression was normalized to GAPDH as housekeeping gene. Relative ratio control was designated as (100%), 1-fold. Data represent $2^{-\Delta\Delta CT}$ values calculated using the $\Delta\Delta C_T$ method. Calculations were shown among Table 2. Relative expression of MOR (mu-OR) on effect of morphine was shown less level than control (0.61-fold) (Figure 1a). Similar finding was also shown with DOR, 0.67-fold, as well as with KOR 0.65-fold (Figures 1 and 2). On the other hand, the relative expression of MOR (mu-OR) did not exhibit any relative elevation but shown less levels under effect of the most opioid treatments (in comparison to control). However, this decrease may due to morphine-induced ORs down-regulation ($p > 0.05$), (Figures 1 and 2). In contrast, it was found that fentanyl treatment caused significantly increased in the mRNA levels of DOR gene (1.71-fold), Figures 1b and 2. Whereas, enkephalin treatment demonstrated highly significantly increased in the mRNA levels of DOR (6.3-fold, $p = 0.002$) (Figures 1b and 2). However, relative expression of KOR was shown slightly increased under effect of both fentanyl and enkephalin treatments (1.04-fold and 1.3-fold, respectively), this increasing was not significant ($p > 0.05$). While, KOR expression was found highly significant increased under effect of Ketazocine (7.16-fold, $p = 0.004$) (Figures 1c and 2).

Table 2: Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase as housekeeping gene

Sample	C_T (mean)	SD	ΔC_T	$\Delta\Delta C_T$	SE	RQ mean	RQ maximum	RQ mean
Control								
MOR	24.5	0.61	4.58	0.0	0.27	0.49	2.0	1.0
DOR	23.0	0.64	3.08	0.0	0.28	0.48	2.1	1.0
KOR	22.7	0.52	2.78	0.0	0.23	0.5	1.9	1.0
Morphine								
MOR	23.68	1.1	3.88	−0.7	0.51	0.26	1.44	0.61
DOR	22.32	0.82	2.52	−0.56	0.36	0.35	1.3	0.67
KOR	21.96	0.47	2.16	−0.62	0.21	0.41	1.02	0.65
Fentanyl								
MOR	23.6	0.46	4.5	−0.08	0.20	0.54	1.65	0.94
DOR	22.96	0.56	3.86	0.78	0.25	0.94	3.13	1.71
KOR	21.94	0.47	2.84	0.06	0.21	0.59	1.83	1.04
Enkephalin								
MOR	23.88	0.56	4.48	−0.1	0.25	0.59	1.46	0.93
DOR	25.14	0.30	5.74	2.66	0.13	4.66	8.57	6.3
KOR	22.56	0.52	3.16	0.38	0.23	0.85	1.99	1.3
Ketazocine								
MOR	23.62	0.45	4.4	−0.16	0.20	0.62	1.28	0.89
DOR	22.56	0.31	3.36	0.94	0.14	0.92	1.6	1.21
KOR	24.82	0.64	5.62	2.84	0.28	4.44	11.53	7.16
GAPDH housekeeping gene								
Control	19.92	0.8						
Morphine	19.8	0.44						
Fentanyl	19.1	0.65						
Enkephalin	19.4	0.30						
Ketazocine	19.18	0.23						

Relative ratio control was designated as (100%), 1-fold. Data represent $2^{-\Delta\Delta CT}$ values calculated by using the $\Delta\Delta C_T$ method. Relative quantity MOR, DOR and KOR gene expression post-exposure to morphine sulfate (Gr1), fentanyl (Gr2), D-penicillamine (2,5) enkephalin (Gr3), and ketazocine (Gr4). MOR: Mu-opioid receptor, DOR: Delta-opioid receptor, KOR: Kappa-opioid receptor, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, SD: Standard deviation, SE: Standard error, RQ: Relative Quantification.

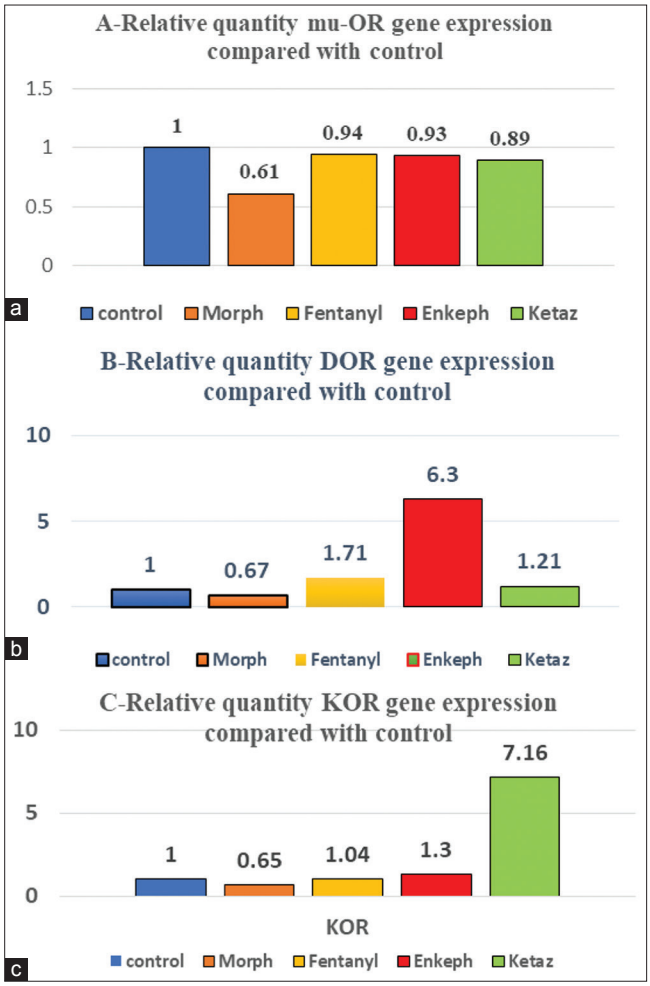

Figure 1: Comparative expression of Mu-OR (a), DOR (b) and KOR (c) genes through exposure to different opioid substances: Morphine sulfate, fentanyl, penicillamine (2,5) enkephalin, and ketazocine for 24 h. Mu-OR: Mu-opioid receptor, DOR: Delta-opioid receptor, KOR: Kappa-opioid receptor

Discussion

Indeed, existence of ORs and opioids peptides in heart myocardial cells may essential for various physiological functions. Where ORs are included three types: mu (MOR), delta (DOR) and kappa (KOR). The levels of such opioid's peptides and ORs can be elevated during episodes of stress, such as ischemia. In addition to heart myocardial cells, delta- and kappa-opioid peptides have been also found in sympathetic never fibers and ganglion cells (reviewed by Feng *et al*., 2012) [16]. In this current study, we have successfully estimated the relative quantity of MOR, DOR, and KOR mRNA levels in HL-1 mouse cardiac myocytes by using specific primers and probes through running qRT-PCR assay. Thus, the results demonstrated that, qRT-PCR is extremely sensitive assay with a high accuracy and wide detection rang. Data analysis of MOR, DOR, and KOR mRNA expression on effect of morphine was shown less level than control (0.61-fold, 0.67-fold, and 0.65-fold, respectively). This finding may confirm that morphine-induced ORs down-regulation and consistent with previous study [17]. In addition, MOR mRNA expression was lowest in the heart tissue [18]. Controversial, morphine effect demonstrated to be specific for KOR; hence, the MOR agonist morphine did not mimic the influences of the KOR agonists [16]. In contrast, it was found that fentanyl treatment caused

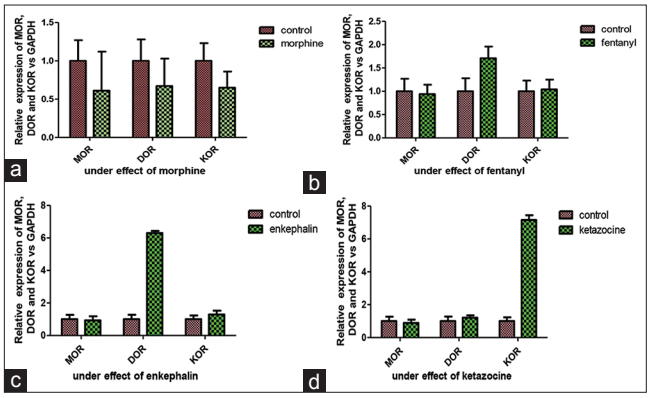

Figure 2: Relative expression of MOR (mu-OR) on effect of morphine was shown less level than control, 0.61-fold (a). Similar finding was also shown with expression of DOR, 0.67-fold (a), as well as with expression of KOR 0.65-fold (a). Whereas, relative expression of DOR on effect of fentanyl was shown increased to 1.71-fold (b). Relative expression under effect of enkephalin treatment demonstrated highly significantly increased in the mRNA levels of DOR, 6.3-fold, p = 0.002) (c). While, KOR expression was found highly significant increased under effect of Ketazocine, 7.16-fold, p = 0.004 (d). Bars, represented standard error. MOR: Mu-opioid receptor, DOR: Delta-opioid receptor, KOR: Kappa-opioid receptor

significantly increased in the mRNA levels of DOR gene (1.71-fold). Therefore, fentanyl may play crucial role preconditioning in reducing ischemia (I)/reperfusion (R) injury in rats [19]. Whereas, enkephalin treatment demonstrated highly significantly increased in the mRNA levels of DOR (6.3-fold, p = 0.002). However, relative expression of KOR was shown slightly increased under effect of both fentanyl and enkephalin treatments (1.04-fold and 1.3-fold, respectively), this increasing was not significant (p > 0.05). Enkephalin that acts to DOR has been mediated in ischemic preconditioning, endogenous opioid-induced preconditioning, and/or reaction to myocardial ischemia in canine [20]. Recently, Eribis peptide 94, a novel enkephalin derivative that binds with high potency to DOR caused an acute reduction in myocardial infarct size [21]. Therefore, some studies support that exogenous administration of DOR agonists may be contributed to be cardioprotective when given acutely pre-ischemia, or immediately pre-reperfusion [22]. Whereas, KOR expression was found highly significant increased under effect of Ketazocine (7.16-fold, p = 0.004). Ketazocine is the exogenous drug with the highest affinity for KOR [5]. However, the KOR is involved in opioid-induced cardioprotection. On the otherwise. Myocardial expression of ORs also evidences changeable upon pathological conditions: Ischemia/reperfusion induces DOR mRNA together with KOR mRNA in myocardial area-at-risk in porcine [23]. The finding of DOR and KOR, but not MOR, receptors in the rat and human heart could, in part, suggested a paracrine role for the opioid produced locally within cardiac tissue. In conclusion, consistent to most studies have found DOR and KOR, but not MOR expressed in cardiomyocytes among several mammalian animals. In HL-1 mouse cardiac myocytes under effect of exogenous opioid analogists exhibited a high affinity to DOR and KOR but not MOR, suggesting the presence DOR and KOR in mouse cardiomyocytes. Thus, cardiomyocytes are capable of synthesis, storage, and release of OR peptides. Actually, exogenous analogist's opioids can mimeses the endogenous analogist's opioids. The further study should be performed on myocardial infarction, ischemia/reperfusion injury.

Ethical Approval

This study was approved by the Ethics Committee of Research Unit, College of Medicine, Qassim University, Saudi Arabia (Ethical approval # 02QUCOM/2020; Date: 2/12/2020).

Availability of Data and Material

The data used in this study are available and will be provided by the corresponding author on a reasonable request.

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