J Anesth (2005) 19:263–265
DOI 10.1007/s00540-005-0327-z

Journal of
Anesthesia

Opioid receptor stimulation does not affect cellular hypoxia-induced gene responses mediated by hypoxia-inducible factor 1 in cultured cell lines

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Abstract
Hypoxia induces a series of adaptive physiological responses including gene inductions. Hypoxia-inducible factor 1 (HIF-1) is a master transcription factor that regulates hypoxia-induced gene expression to maintain homeostasis in the living body. Opioids are potent analgesic agents that are widely used in clinical practice. Therefore, we investigated the effect of opioids on HIF-1 activity. SH-SY5Y human neuronal cells, which express opioid receptors intrinsically, were cultured under 1% or 20% O_2 conditions with or without treatment by DAGO, DPDPE, or U-50488, which are the selective agonists of μ-, δ-, and κ-opioid receptors, respectively. Expression of subunits of HIF-1, HIF-1α, and HIF-1β were examined by Western blot using specific antibodies. Expression of the HIF-1-dependent gene were investigated by reporter assay. None of the selective agonists of opioid receptors tested affected HIF-1 activation by hypoxia. Therefore, it is suggested that opioid receptor-mediated signals do not affect HIF-1-dependent cellular hypoxia-induced gene responses.

Key words Hypoxia · Hypoxia-inducible factor 1 (HIF-1) · Opioid · Guanine nucleotide-binding (G) protein-coupled receptor (GPCR) · SH-SY5Y

Hypoxia induces a series of adaptive physiological responses. The carotid body and neuroepithelial body are special sensor organs that detect O_2 concentration in the bloodstream. Today it is appreciated that all the cells have their own oxygen-sensing system to elicit gene expression mediated by transcription factors to accommodate hypoxia [1]. Hypoxia-inducible factor 1 (HIF-1) is a master transcription factor that regulates hypoxic gene expression and maintains cellular energy homeostasis. In addition to energy metabolism, activated HIF-1 induces expression of its target genes required for angiogenesis, erythropoiesis, cell proliferation and viability, vascular remodeling, and vasomotor responses [1]. HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β subunits that bind to the specific regulatory sequences known as the hypoxia response element (HRE). There is an established consensus that the biological activity of HIF-1 is mainly determined by the expression and transcriptional activity of the HIF-1α subunit [2]. The HIF-1α protein expression level is determined by an elaborate balance between protein synthesis and degradation as well as other intracellular proteins. The substrate (O_2) limitation induces HIF-1α protein stabilization and transcriptional activation without induction of the mRNA [3].

HIF-1 activity is governed not only by O_2 tension but by various kinds of stimulation. We previously reported that insulin-like growth factor 1 (IGF-1) [4], prostaglandin E_2 (PGE_2) [5], the nitric oxide donor NOC18 [6], and stimulation of M1 or M3 subtypes of muscarinic acetylcholine receptor induce HIF-1 activation under normoxic conditions [7].

Because HIF-1 mediates physiological and pathophysiological responses with adaptive changes in target gene expression, it is intriguing to examine the effect of drugs used during the perioperative period on HIF-1 activity. We have reported the effect of the volatile anesthetic halothane [8], the intravenous anesthetic propofol [9], and local anesthetics [10] on hypoxia-induced HIF-1 activation in established cell lines. There have been no reports yet on the effect of opioids on the hypoxia-induced gene responses mediated by HIF-1 to the best of our knowledge. In this study we focus on opioids, which are potent analgesic agents and are widely used clinically for anesthesia and in pain clinics.

We examined the effect of opioids on the accumulation of HIF-1α (Fig. 1, top panel). Phosphorylation of

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Received: November 30, 2004 / Accepted: April 4, 2005

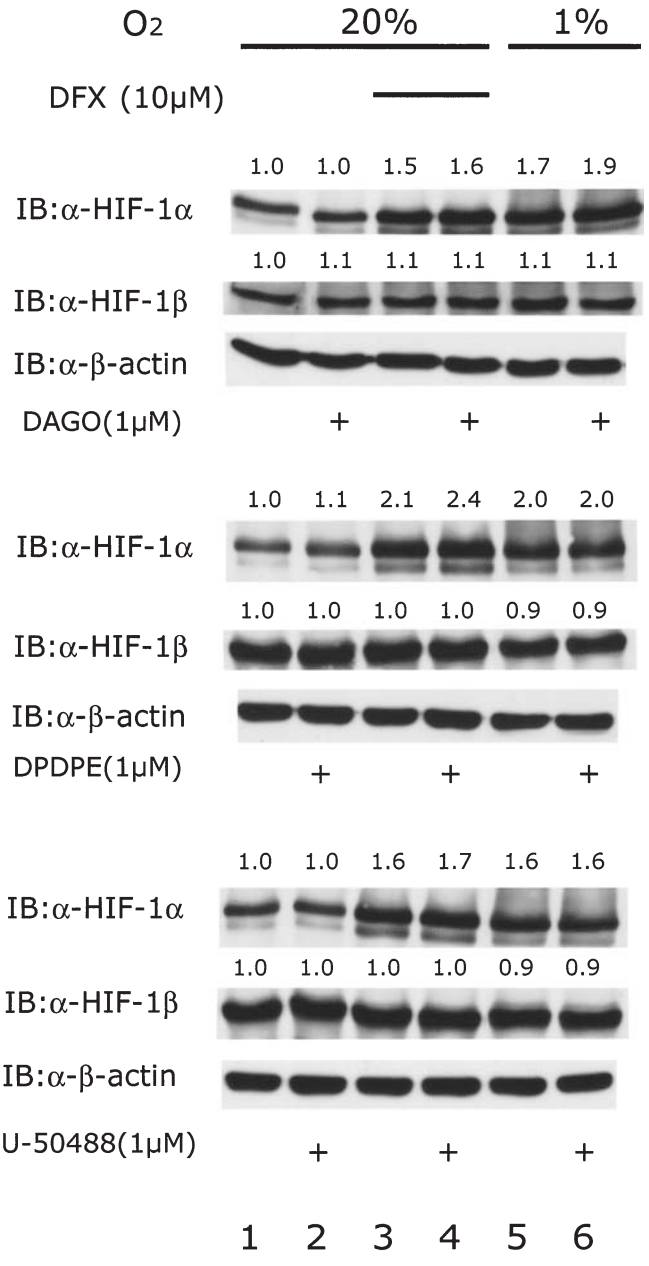

Fig. 1. Effects of selective opioid agonists on hypoxia-inducible factor 1 (HIF-1α) accumulation in SH-SY5Y cells. SH-SY5Y cells were treated with [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAGO), [D-Pen^{2,5}]-enkephalin (DPDPE), or (±)-*trans*-U-50488 methanesulfonate salt (U-50488) at 1 μM (lane *2*); or with 10 μM DFX (lane *4*) under 20% O_2 or 1% O_2 (lane *6*) for 4 h. They were then harvested for immunoblot assays using anti-HIF-1α, anti-HIF-1β, or anti-β-actin antibody. In lane *1* the cells underwent no treatment. We performed the experiment under almost the same conditions more than three times. The data presented are representative of the results of those experiments. The protein was quantified by densitometry assisted by the image analysis software NIH Image (version 1.62) and indicated as fold induction to each lane *1* after normalization to the value of expression for β-actin of the corresponding lane [6, 8, 11]

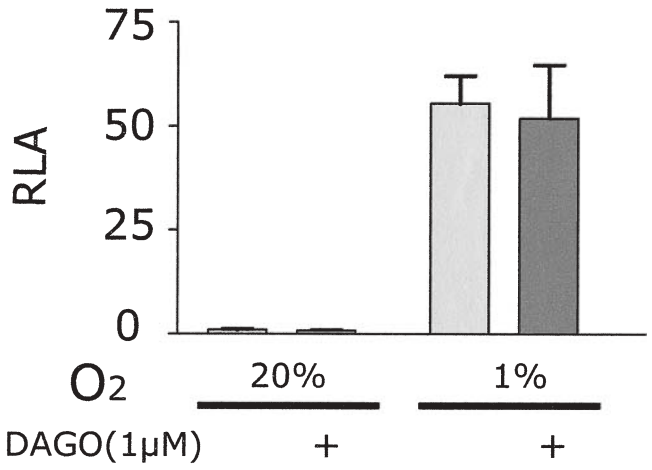

Fig. 2. Effects of *DAGO* in normoxia or hypoxia-induced HRE-dependent gene expression. SH-SY5Y cells were transfected with 50 ng of pRL-SV40 (control reporter encoding *Renilla* luciferase) and 200 ng of p2.1 (HRE-driven reporter encoding firefly luciferase) using Fugene 6 transfection reagent (Roche) [6, 7]. After 6 h of incubation, cells were treated with DAGO under 20% or 1% (*dark lines*) O_2 for 18 h and harvested for luciferase assays. The ratio of firefly/*Renilla* luciferase activity was determined and normalized to the value obtained from untreated cells under 20% O_2 to obtain the relative luciferase activity (*RLA*) [6, 7]

ERK1 and ERK2 were observed after treatment with [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAGO), [D-Pen^{2,5}]-enkephalin (DPDPE), or (±)-*trans*-U-50488 methanesulfonate salt (U-50488), indicating that SY5Y cells express μ, δ, and κ opioid receptor functionally (data not shown). SH-SY5Y cells express HIF-1α under 20% O_2 condition (lane 1) without treatment with opioids; the expression increases in response to the iron chelator DFX (lane 3) and exposure to 1% O_2 atmosphere (lane 5), both of which are identified as strong HIF-1α inducers [6, 8, 11]. In contrast, all of the selective agonists of opioid receptors used in this study had no effect of the accumulation of HIF-1α under the above-mentioned conditions (lanes 2, 4, and 6). Expression of HIF-1β was constant (Fig. 1, middle panel). The expression of β-actin was not affected by the treatments (bottom panel). Expression of the mRNA of HIF-1α and HIF-1β was not affected significantly (data not shown).

Next, we investigated the effects of opioids on HIF-1 activation using a reporter gene system (Fig. 2). SH-SY5Y cells were transfected with the reporter p2.1 containing HIF-1-dependent HREs and pSV40-RL encoding *Renilla* luciferase, incubated with or without DAGO, and then subjected to a reporter assay, which is the most sensitive assay for examining HIF-1 activation. Hypoxic treatment increased HRE-dependent gene expression about 60-fold. DAGO did not affect hypoxia-induced HRE-dependent gene expression or

expression under 20% O_2 conditions, suggesting that activation of μ-opioid receptor had no effect on HIF-1-dependent gene expression. This result is consistent with our unpublished observation that stimulation of the receptors did not affect HIF-1 activity using the Chinese hamster ovary (CHO)-derived established cell line CHO-1 stably exogenously expressing the μ-, δ-, or κ-opioid receptors.

Using SH-SY5Y human neuroblastoma cells, we demonstrated the null effect of opioid receptor stimulation on hypoxia-mediated gene expression mediated by the transcription factor HIF-1. Opioid receptor-mediated signals affect neither protein expression of HIF-1 nor gene expression tested by reporter gene assay under normoxic or hypoxic conditions. It is reported that opioid receptors constitute mono- and hetero-complexes with each other, and these new receptor complexes exhibit functional properties that are distinct from those of either receptor [12]. Moreover, opioid receptors (1) functionally and physically cross-talk with other guanine nucleotide-binding (G) protein-coupled receptors, such as β-adrenergic receptors, via multiple hierarchical mechanisms including heterodimerization of these receptors, (2) counterbalance functional opposing G protein signaling, and (3) interface at a downstream signal [13]. Further studies are to be performed especially in situations similar to clinical settings, such as evaluating the coexistence of opioid analgesics and catecholamines.

Opioids are widely used as analgesics in combination with general anesthetics or tranquilizers in various clinical settings. The receptors are expressed not only in neuronal cells but also immunocompetent cells. Because HIF-1 is a key molecule for the cells to adapt to hypoxic conditions, activation of HIF-1 may play an important protective role in critical organs. In fact, experiments using genetically modified mice clearly indicate a critical role for HIF-1 in organ protection against hypoxic insults. Taking into account evidence that the volatile anesthetic halothane [8] and the intravenous anesthetic propofol [9] inhibit HIF-1 activity, patient management under general anesthesia or in intensive care using opioid receptor ligands may have some advantage from this point of view. Further extensive studies are required to examine the effects of opioid analgesics used in the clinical setting, as each drug acts on multiple subtypes of receptors and sets up interactions among them.

Acknowledgments. We thank Dr. Gregg L. Semenza, Johns Hopkins University, for providing reporter plasmids. This study was supported by research grants from the Ministry of Science, Education, and Sport to T.A. and K.H.

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