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RESEARCH ARTICLE

Curcumin induces differentiation of embryonic stem cells through possible modulation of nitric oxide-cyclic GMP pathway

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ABSTRACT

Curcumin, an active ingredient of dietary spice used in curry, has been shown to exhibit anti-oxidant, anti-inflammatory and anti-proliferative properties. Using EB directed differentiation protocol of H-9 human embryonic stem (ES) cells; we evaluated the effect of curcumin (0–20 μmol/L) in enhancing such differentiation. Our results using real time PCR, western blotting and immunostaining demonstrated that curcumin significantly increased the gene expression and protein levels of cardiac specific transcription factor NKx2.5, cardiac troponin I, myosin heavy chain, and endothelial nitric oxide synthase during ES cell differentiation. Furthermore, an NO donor enhanced the curcumin-mediated induction of NKx2.5 and other cardiac specific proteins. Incubation of cells with curcumin led to a dose dependent increase in intracellular nitrite to the same extent as giving an authentic NO donor. Functional assay for second messenger(s) cyclic AMP (cAMP) and cyclic GMP (cGMP) revealed that continuous presence of curcumin in differentiated cells induced a decrease in the baseline levels of cAMP but it significantly elevated baseline contents of cGMP. Curcumin addition to a cell free assay significantly suppressed cAMP and cGMP degradation in the extracts while long term treatment of intact cells with curcumin increased the rates of cAMP and cGMP degradation suggesting that this might be due to direct suppression of some cyclic nucleotide-degrading enzyme (phosphodiesterase) by curcumin. These studies demonstrate that polyphenol curcumin may be involved in differentiation of ES cells partly due to manipulation of nitric oxide signaling.

KEYWORDS curcumin, nitric oxide, cyclic GMP, embryonic stem cells

INTRODUCTION

Curcumin, a yellow pigment from the root-bearing *Curcuma longa* Linn plant, is a member of ginger family. It is a natural polyphenolic compound that has been used in alternative medicine to treat common ailments associated with injury and inflammation (Lodha and Bagga, 2000; Singh, 2007). A vast number of studies have shown that curcumin exhibits activity against bacterial, viral and fungal infections and it exhibits anti-proliferative, anti-inflammatory and pro-apoptotic effects (Singh and Aggarwal.1995; Aggarwal et al., 2007; Aggarwal and Sung, 2008).

Embryonic stem cells (ES) and more recently induced pluripotent stem cells have been thought to revolutionize field of regenerative medicine due to their two unique properties, self-renewal and pluripotency (Mujoo et al., 2011). Previous studies have indicated that cucumin plays an important role in

regulation of cell differentiation. For instance, vitamin E and other antioxidants including curcumin induce differentiation of HL-60 premyelocytic leukemia in combination with vitamin D3 (Sokoloski et al., 1997). In contrast, curcumin has been shown to inhibit angiogenic differentiation of human umbilical vein endothelial cells *in vitro* and angiogenesis in a subcutaneous matrigel plug model in mice (Thaloor et al., 1998). Additional studies demonstrate that curcumin is involved in stimulation of muscle regeneration after traumatic injury by directly inducing proliferation and differentiation of muscle precursor cells (Thaloor et al., 1999). Furthermore, curcumin has been shown to protect endothelial dysfunction by possible regulation of heme-oxygenase-1 (HO-1) and guanylyl cyclase pathway (Fang et al., 2009). Earlier studies have also shown that curcumin induces neurogenesis, synaptogenesis and migration of neural progenitor cells *in vitro* and *in vivo* (Kang et al., 2006; Kim et al., 2008). Since curcumin has been shown to be involved in regulation of cell proliferation and differentiation, we were interested in elucidating the role of curcumin in differentiation of ES cells and evaluating the molecular mechanism(s) of such differentiation.

Nitric oxide-cyclic GMP pathway mediates important physiological functions in cardiovascular and nervous system. At the cellular level, NO-cGMP pathway is involved regulation of cell proliferation and differentiation. The important role of NO-cGMP pathway in regulation of stem cell differentiation has emerged from our studies and that of other investigators (Mujoo et al., 2011). Our previous studies demonstrate differential expression and function of various nitric oxide signaling components in embryonic stem cells and differentiated cells (Krumenacker et al., 2006; Mujoo et al., 2006). Furthermore, we have shown that both nitric oxide donors and soluble guanylyl cyclase (NO receptor) activators demonstrate enhanced differentiation of human and mouse embryonic stem cells and combined effects of these agents were additive. This was accompanied by robust induction in cGMP production compared with either of the two agents alone suggesting that NO-cGMP pathway plays a significant role in differentiation of stem cells (Mujoo et al., 2008).

Studies from other investigators indicate that NO-cGMP pathway has been implicated in the differentiation of stem cells into cells of various lineages in response to various plant compounds. For instance, Zhu et al. (2006) demonstrated that icariin (a constituent of Epimedium, a traditional Chinese medicine) induced differentiation of mouse ES cells into cardiomyocytes by elevation of cAMP/cGMP ratio in ES cells as well as up regulation of the endogenous generation of NO during the early stages of cardiac development. Similarly, the plant compound genistein has been shown to stimulate osteoblastic differentiation in bone marrow culture via the NO-cGMP pathway (Pan et al., 2005). Therefore, our objectives for current study were to determine the role of curcumin in differentiation of ES cells and examine the possible role of nitric oxide signaling pathway in mediating such differentiation.

RESULTS

Effect of curcumin in ES cell differentiation

Our results demonstrate that exposure of partially differentiated stem cells (EB stage, day 7) to various concentrations of curcumin increased the expression of cardiac specific transcription factor NKx2.5 in both mouse (2-fold increase in NKx2.5 relative to vehicle control; data not shown) and human ES (H-9) cells. Fig. 1 demonstrates that when partially

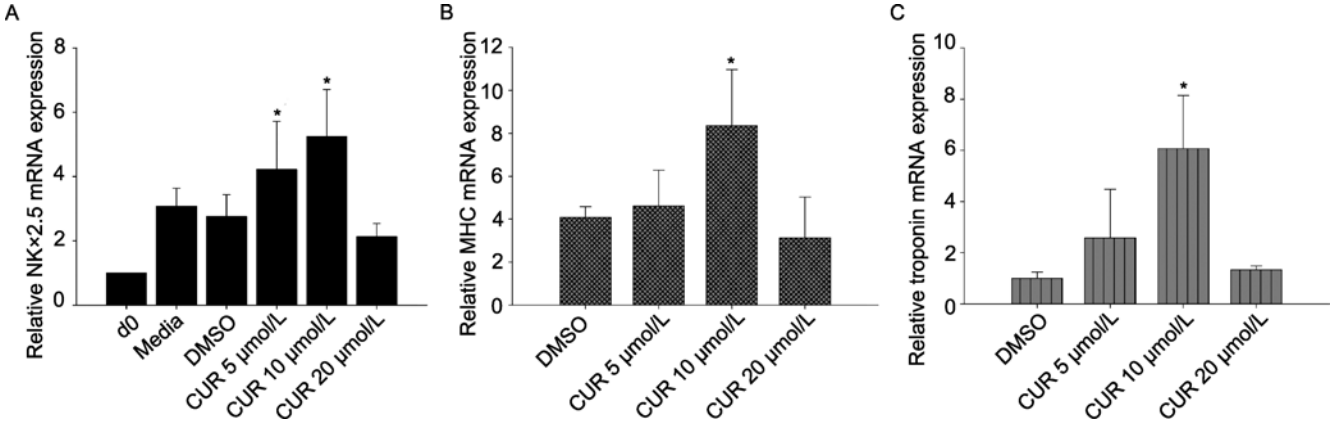

Figure 1. Effect of curcumin in H-9 human ES cells. Embryonic Stem cells (H-9; d0) cells were subjected to embryoid body (EB) directed differentiation and partially differentiated cells (EB stage; d6) were exposed to various concentrations of curcumin (5–20 μmol/L) on day 7, 9, 11 and 13. Cells were harvested on day 14 and samples were analyzed for gene expression of NKx2.5 (A), troponin I (B), myosin heavy chain (C) using real time PCR normalized to housekeeping gene GAPDH and presented as fold expression compared to day 0. Day 0 represents undifferentiated cell culture (H-9) collected prior to subjecting cells to differentiation. The data were analyzed using the $_2^{-\Delta\Delta C}{}_T$ method. Error bars indicate ±SEM. * indicates significance using paired students *t*-test. * $P < 0.05$ between DMSO control vs. various treatment groups; $n = 3–9$. DMSO (0.01%) was used as a vehicle control.

Table 1 Intracellular nitrite levels in response to curcumin in the extracts of differentiated H-9 human embryonic stem cells. Data are mean ± SEM (n = 3).

Assay conditions	Nitrite (μmol/L per mg protein)	Fold increase relative to DMSO
DMSO	0.514 ± 0.15	1
5 μmol/L curcumin	0.62 ± 0.10	1.2
10 μmol/L curcumin	0.903 ± 0.16	1.75
10 μmol/L NOC-18	0.909 ± 0.20	1.77
NOC-18 + curcumin	1.437 ± 0.39	2.79

differentiated cells (H-9) were exposed to various concentrations and multiple treatments (day 7, 9, 11 and 13) of curcumin (5–20 μmol/L), 10 μmol/L concentration of curcumin significantly increased the mRNA expression of cardiac specific transcription factor NKx2.5 by 47% (A) and 2–3 folds increase in the expression of cardiac troponin I and myosin heavy chain (Fig. 1B and 1C). This was associated with a modest decrease in NOS-2 mRNA expression compared to the cells treated with media or DMSO alone. Interestingly, we observed a 3.4-fold increase in mRNA expression of endothelial nitric oxide synthase (NOS-3) in cells treated with 10 μmol/L curcumin (data not shown). Higher concentrations of curcumin (20 μmol/L) were slightly toxic to the cells (based on decreased cell growth and detachment of the cells from the substrate), which may explain a decrease in gene expression of various markers such as NKx2.5 and others at this concentration.

In order to demonstrate the effects of curcumin were due to an increase in NO production or signaling, we analyzed both the surrounding media and cell lysates for nitrite levels. Table 1 demonstrates that incubation of cells with curcumin led to a dose dependent increase in intracellular nitrite to the same extent as giving an authentic NO donor (Fold increase relative to DMSO: 5 μmol/L curcumin, 1.2-fold; 10 μmol/L curcumin, 1.75-fold; NOC-18, 1.77-fold; NOC + curcumin 2.79-fold). However, part of the increase in intracellular nitrite may also be due to cellular uptake from the surrounding media since the media nitrite was lower when incubated with curcumin. There was no detectable nitrite in the curcumin solution added to the cells.

Combination of curcumin and an NO donor in human ES cell differentiation

Since we observed induction of NOS-3 gene with curcumin alone, we were therefore interested in investigating the combined effect of curcumin and activators of NO signaling pathway in ES differentiation. Our studies (Fig. 2A) demonstrate that combination of NO donor NOC-18 and curcumin (multiple treatments) showed a modest increase in the expression of NKx2.5 (2.85-fold) compared to NOC-18 (1.5-fold) or curcumin (2.5-fold) separately. Although there was an increase (1.5-fold) in the expression of cardiac specific transcription factor NKx2.5 when the cells were exposed to turmeric (yellow spice used in curry which contains mixture of compounds including curcumnoids), combination of turmeric + NOC-18 did not further induce the gene expression of NKx2.5 suggesting that a critical concentration of curcumin (active ingredient of turmeric) may be required for the additive effect in mediating differentiation of stem cells under these conditions. When same samples were analyzed for sGC α_1 and NOS-2 expression (Fig. 2B), there was a modest decrease in NOS-2 expression with curcumin. Turmeric alone also exhibited inhibition of NOS-2 expression. Our results further indicate an increase (>49%) in sGC α1 gene expression when partially differentiating cells were exposed to combination of NOC-18 and curcumin relative to NOC-18 (5%) and curcumin (25%) separately. Similarly, when EBs (day 1 and day 2; early stage) were exposed to curcumin, NOC-18 or the combination, there was a 2.33-fold increase in the NKx2.5 gene expression (Fig. 2C) compared to curcumin (1.87-fold increase) or NOC-18 (1.14-fold increase) alone. When same samples were further analyzed for sGC β1 expression (Fig. 2D) there was a 44% increase in the gene expression in cells treated with NOC-18 + curcumin, compared to 22%–28% increase in sGC β1 expression in cells treated with NOC-18 or curcumin alone suggesting that components of nitric oxide pathway may be involved in curcumin-induced differentiation of stem cells.

Effect of curcumin and sGC activator in human ES cell differentiation

Results of Fig. 3 demonstrate that although curcumin and allosteric sGC activator BAY41-2272 significantly induced the expression of NKx2.5 by 2.18–2.7 folds respectively, whereas their combination did not further enhance the differentiation of stem cells into myocardial cells (Fig. 3A). Combination of curcumin and BAY41-2272 modestly increased expression of NOS-3 (2-fold increase relative to DMSO) compared to curcumin (1.5-fold) and BAY 41 (1.8-fold) alone (Fig. 3B). Similarly submaximal concentrations of a cell permeable cGMP analog, 8-bromo-cGMP, and curcumin did not further enhance the levels of cardiac specific transcription factor Nkx2.5 suggesting that a cGMP-independent pathway may also be involved in curcumin-induced differentiation of stem cells into cells of mixed lineage (data not shown).

Immunostaining of marker proteins in response to curcumin

To further investigate the effect of curcumin in mediating the

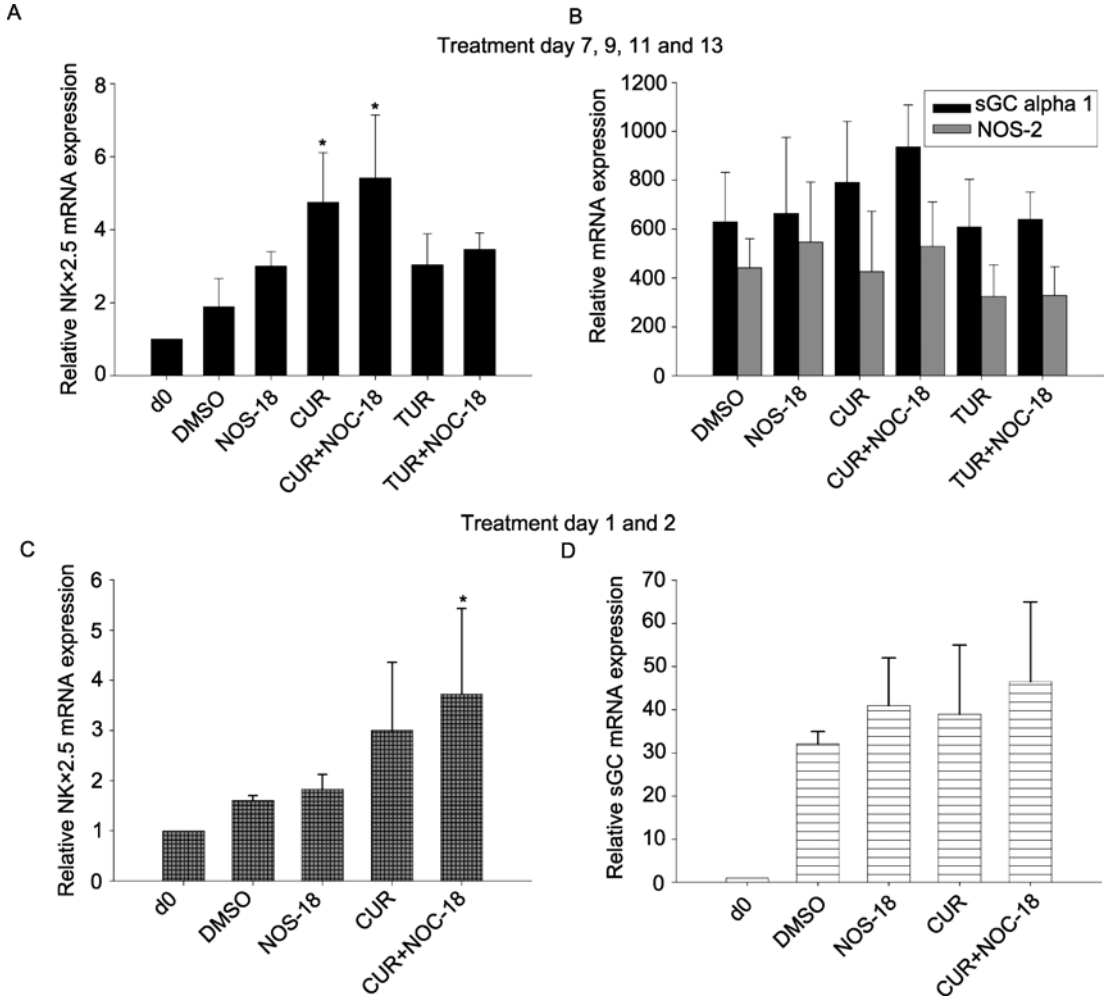

Figure 2. Combination of NO donor and curcumin in H-9 cells. Embryonic stem cells (H-9; d0) cells were subjected to embryoid body (EB) directed differentiation and partially differentiated cells (EB stage; d6) cells were exposed to multiple treatment of NO donor NOC-18 (5 μmol/L), curcumin (10 μmol/L) and the combination of the two (NOC + curcumin), turmeric (10 μmol/L), or NOC + turmeric combination on day 7, 9, 11 and 13. Cells were harvested on day 14 and samples were analyzed for Nkx2.5 (A) and sGC α_1 and NOS-2 transcript levels (B). The EBs in C and D were exposed to NOC-18 (10 μmol/L), curcumin (10 μmol/L) or their combination on day 1 and 2 of differentiation and the indicated genes were analyzed by quantitative PCR (Error bars indicate ±SEM. * indicates significance using paired students *t*-test, DMSO vs. treatment groups). * $P < 0.05$; $n = 5–7$.

differentiation of stem cells at the protein level, we conducted immunofluorescent staining of marker proteins in differentiated cells exposed to curcumin and turmeric. Our results indicate that when differentiating EBs were exposed to curcumin or turmeric (10 μmol/L), NOC-18 (5 μmol/L) or the combination of NOC + curcumin and NOC + turmeric staining of cardiac specific protein myosin light chain 2 (MLC2) and smooth muscle actin (SMA) was enhanced in differentiating cells compared to vehicle controls. Compared to other treatment groups curcumin showed both cytoplasmic and filamentous staining. In contrast, turmeric exhibited enhanced cytoplasmic staining of differentiated cells (Fig. 4A and 4B). Our results also demonstrated enhanced beating area(s) in differentiated H-9 cells exposed to curcumin or turmeric compared to DMSO control. Fig. 4C shows photographs of beating area(s) of H-9 differentiated cells exposed to DMSO, curcumin or turmeric. These results collectively demonstrate that curcumin induces differentiation of stem cells into cells of mixed lineage including functional myocardial cells.

Effect of curcumin on p53/p21 pathway and ERK phosphorylation using western blot

In order to elucidate the molecular mechanism(s) involved in differentiation of stem cells by curcumin, we examined the effect of curcumin or its combination with NO donor NOC-18 on p53/p21 pathway. Fig. 5 demonstrates that although slow release NO donor NOC-18 (5 μmol/L) and curcumin

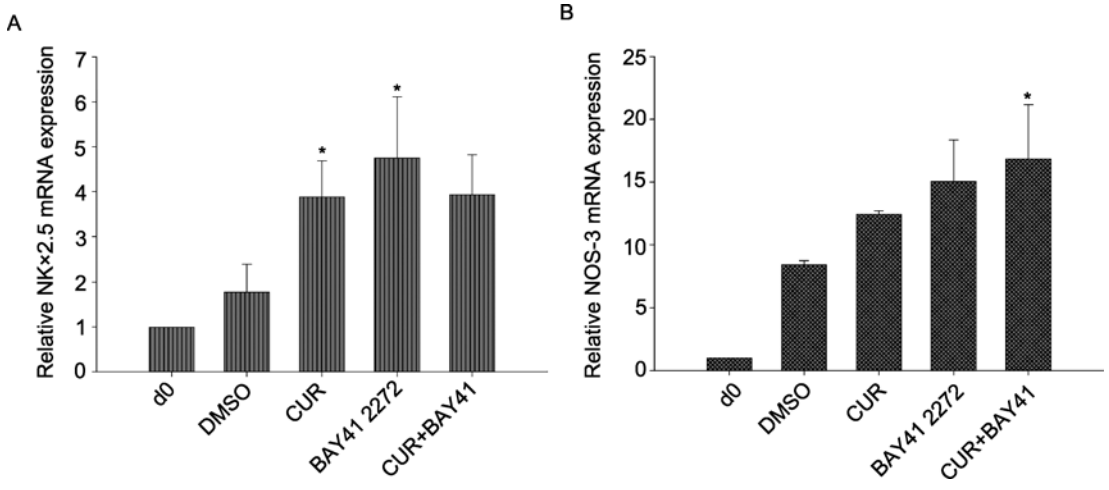

Figure 3. Effect of curcumin and sGC activator BAY41-2272 in H-9 human ES cells. H-9 human embryonic stem cells were subjected to EB directed differentiation and partially differentiated cells (d6) were exposed to multiple treatments of curcumin (10 μmol/L), sGC activator BAY41-2272 (10 μmol/L) or curcumin + BAY41 2272 on day 7, 9, 11 and 13. Cells were harvested on day 14 and the samples were analyzed for Nkx2.5 (A) and NOS-3 (B) genes using real time PCR. Day 0 represents undifferentiated cell culture usually collected prior to subjecting cells to differentiation. The data were analyzed using the $_2{}^{-\Delta\Delta C}{}_T$ method. (Error bars indicate ± SEM. * indicates significance using paired students *t*-test, DMSO vs. treatment group). * $P < 0.05$; $n = 6–9$.

(10 μmol/L) showed modest increase in transcription factor p53, combinations of the two exhibited a further increase (3–4 folds induction) in p53 protein levels as compared to NOC-18 or curcumin alone. An allosteric sGC activator BAY41-2272 also showed a 2–3 folds increase in p53 levels but the combination of curcumin + BAY41-2272 did not further enhance p53 protein levels. Furthermore, cardiac specific protein MLC2 also showed enhanced induction in response to curcumin + NOC-18 in differentiated cells. Combinations of curcumin + NOC-18 and curcumin + BAY41-2272 induced an increase in eNOS protein levels further confirming the data observed by real time PCR (Fig. 3A). Our additional studies demonstrate that cyclin-dependent kinase inhibitor p21/WAF1 protein was also up regulated (2–3 folds relative to curcumin or NOC alone) in cells exposed to a combination of curcumin + NOC-18. Modest up regulation of p21 protein was also observed in cells exposed to a combination of BAY41 + curcumin (Fig. 5A). An allosteric sGC activator YC-1 either alone or in combination with NOC-18 also promoted increase in the levels of p21 protein (Fig. 5B). Our results further demonstrate that curcumin alone similar to NO donor NOC-18, sGC activator BAY41-2272 or cGMP analog 8-bromo-cGMP induced the protein levels of cardiac troponin I (Fig. 5C). These results demonstrate that induction of NOS-3 and stabilization of p53/p21 pathway may be involved in enhanced differentiation of stem cells on exposure of stem cells to combination of NOC-18 + curcumin. Furthermore, our data indicate that curcumin is able to inhibit the phosphorylation of ERK in H-9 differentiated cells compared to cells exposed to either DMSO or NOC-18 (Fig. 5D).

Effect of curcumin on cyclic nucleotide contents and degradation

We were further interested in evaluating whether curcumin will influence the differentiation of stem cells via a mechanism associated with second messengers cAMP or cGMP. Therefore, baseline levels of cAMP and cGMP were measured in cells harvested from the culture. Results shown in Table 2 indicate that continuous presence of curcumin at 10 μmol/L concentration in the cultures of partially differentiated stem cells induced a decrease in the baseline contents of cAMP and significantly elevated baseline contents of cGMP. These changes might result from the influence of curcumin on degradation of cAMP or cGMP. To examine this hypothesis, we have prepared the extracts of the cells and results shown in Table 3 demonstrate that long-term presence of curcumin in the partially differentiated cultures of human ES cells substantially increase the rate of cAMP and cGMP degradation in the extracts by 27% and 25%, respectively. However, curcumin added directly to the extracts had an opposite effect and significantly suppressed cAMP and cGMP degradation by 16%–38%. This might be due to direct suppression of some cyclic nucleotide-degrading enzymes (phosphodiesterase) by curcumin.

DISCUSSION

Previous studies have shown that phytochemical curcumin exhibits diverse biological and pharmacological properties and effects of which have been studied in cardiovascular

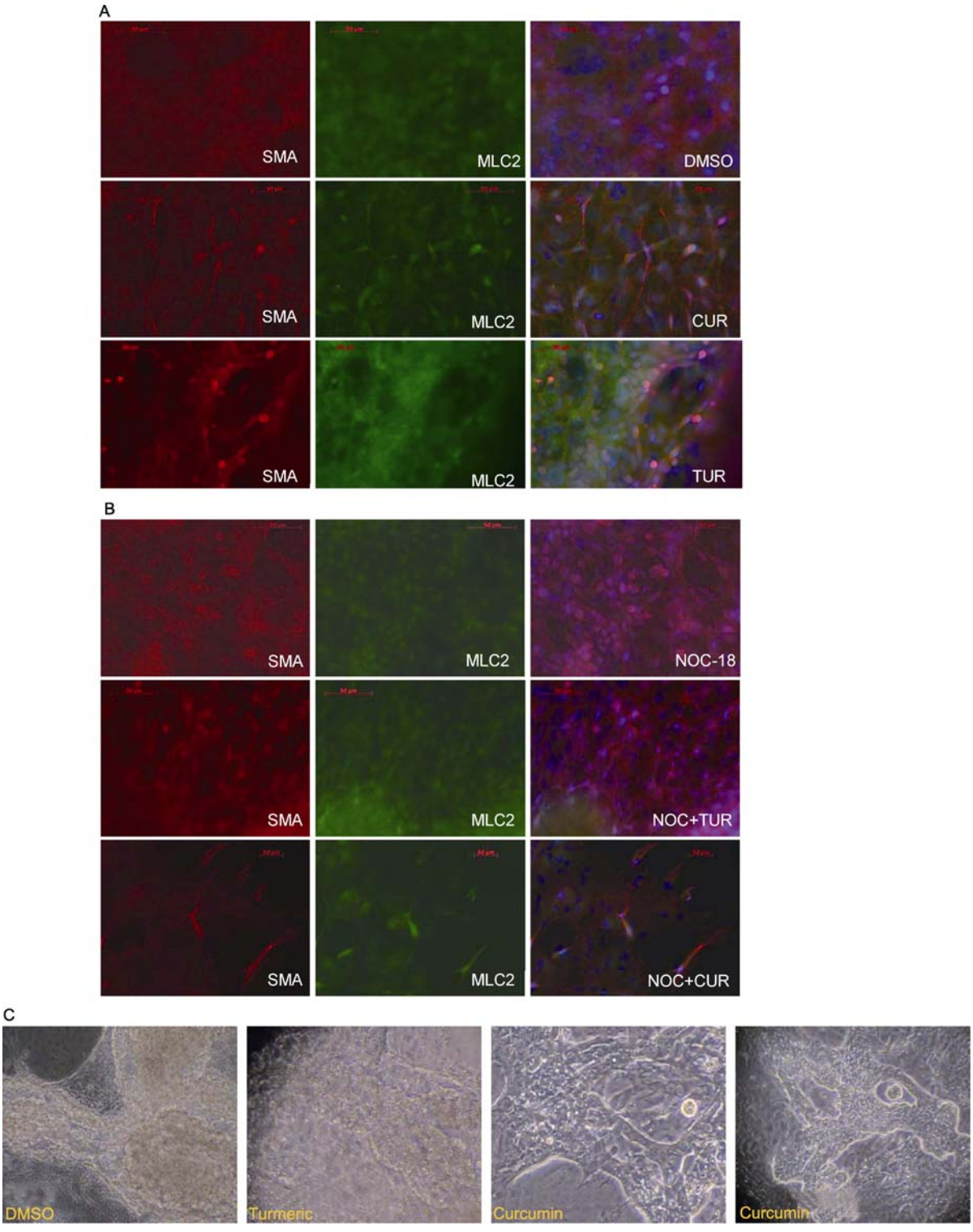

Figure 4. Immunofluorescence detection of MLC2 and smooth muscle actin in H-9 cells exposed to curcumin and turmeric. Differentiated H-9 cells (d13) were exposed to curcumin (10 μmol/L), turmeric (10 μmol/L) or DMSO (0.01%) (A) and NOC-18 (5 μmol/L) , NOC + curcumin and NOC + turmeric (B) for 24 h at 37°C and the cells were fixed with paraformaldehyde and incubated with antibodies to MLC2 (green) and SMA (red) followed by detection with fluorescent conjugated secondary anti-mouse or rabbit antibodies (20× magnification). The cells were counterstained with DAPI for DNA and images were captured with an inverted fluorescent microscope at the scale bar of 50 μm. Figures are representative of 3 independent experiments. (C)The still pictures of contracting (beating) area(s) of myocardial cells exposed to vehicle control, turmeric and curcumin.

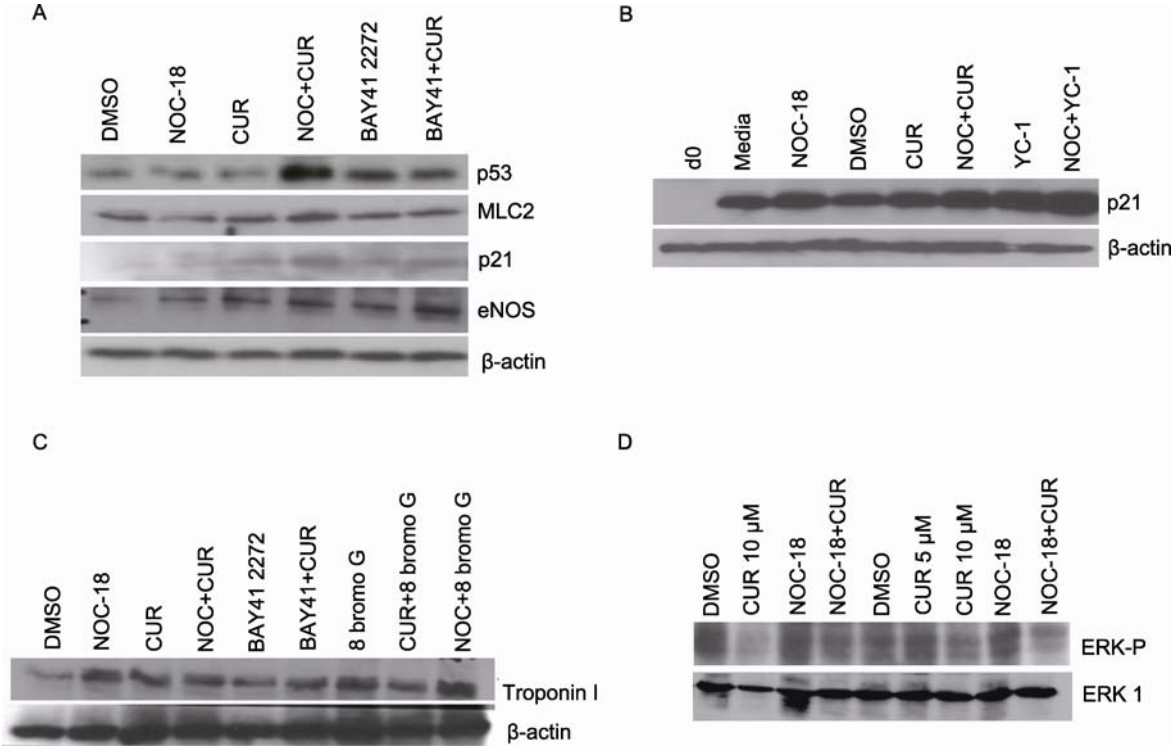

Figure 5. Protein analyses of H-9 cells exposed to curcumin and activators of NO pathway. H-9 human embryonic stem cells were subjected to EB directed differentiation and partially differentiated cells (d6) were exposed to multiple treatments (d7, 9, 11 and 13) of curcumin (10 μmol/L), NOC-18 (10 μmol/L; NO donor), sGC activators BAY 41-2272 or YC-1 (5 μmol/L), cGMP analog 8-bromo-cGMP (0.75 mmol/L) or various combinations as indicated. Equal amount (30 μg) of protein was resolved on SDS-PAGE and membranes were probed by p53, p21, eNOS, MLC2, troponin I, phospho ERK, and total ERK antibodies (A–D). β-actin was used as endogenous control and the proteins were visualized by enhanced chemiluminescence. A representative blot of three independent experiments is shown here. Collectively, our data demonstrates that curcumin induces differentiation of stem cells; however nitric oxide donor further enhanced the differentiation through modulation of NO signaling pathway.

Table 2 Effect of curcumin on baseline cyclic AMP and cyclic GMP contents of differentiated H-9 human embryonic stem cells. Data are mean ± SEM (n = 6–12).

Growth conditions	cAMP (pmol/mg protein)	cGMP (pmol/mg protein)
Vehicle (0.1% DMSO)	3.43 ± 0.17	0.32 ± 0.02
Curcumin (5 μmol/L)	2.81 ± 0.32	0.31 ± 0.02
Curcumin (10 μmol/L)	2.62 ± 0.25*	0.39 ± 0.02*

*P < 0.05.

Table 3 Effect of curcumin on the rates of cyclic AMP and cyclic GMP degradation in the extracts of differentiated H-9 human embryonic stem cells. Data are mean ± SEM (n = 4–8).

Growth condition	Assay condition	cAMP degradation (pmol/min per mg)	cGMP degradation (pmol/min per mg)
Control	Vehicle	155 ± 10	13.2 ± 0.3
	Curcumin	125 ± 8	9.7 ± 1.2[a]
Curcumin	Vehicle	197 ± 9[a]	16.5 ± 0.9[b]
	Curcumin	122 ± 8[b]	13.9 ± 1.3

[a]P < 0.05 versus vehicle control.
[b]P < 0.05 versus extracts of control cells grown in the absence of curcumin.

disease, inflammation, cancer, diabetes, and neurodegenerative diseases (Aggarwal et al., 2007). The role of curcumin in cardiovascular system is well documented as curcumin has been shown to mediate protective effects in isoproterenol

induced myocardial infarction and prevent heart failure in rats due to inhibition of p300 histone acetlytransferase activity (Kim et al., 2008; Morimoto et al, 2008). The effect of curcumin in proliferation and differentiation of cells has also been studied earlier. One such study demonstrated that adult multipotent neural progenitor cells predominantly differentiate into neurons in presence of curcumin (Kang et al., 2006). Additional studies suggest that curcumin is capable of preventing the death of neurons in animal model of neurodegenerative disorders by stimulating the proliferation of embryonic neural progenitor cells and neurogenesis of adult hippocampus via activation of extracellular signal regulated kinases (ERKs) and p38 kinases (Kim et al., 2008). Furthermore, curcumin has been reported to induce differentiation in HL-60 premyelocytic leukemia cells with low concentrations of vitamin D3 and other vitamin analogs which share receptor binding properties with vitamin D3 (Sokoloski et al., 1997) or by directly inducing proliferation and differentiation of muscle precursor cells (Thaloor et al., 1999) after traumatic injury. Our previous studies using ES cells indicate differential expression and function of various NO signaling components (Krumenacker et al., 2006; Mujoo et al., 2006) in ES and differentiated cells. Using activators of NO signaling pathway, we were able to show enhanced differentiation of both mouse and human ES cells with combination of nitric oxide donors (NO donors) and soluble guanylyl cyclase (NO receptor) activators compared to either of the two agents used separately (Mujoo et al., 2008). Nitric oxide mediates important physiological functions associated with integrative body systems including cardiovascular and nervous system, (Murad, 2006; Mujoo et al., 2011). Our current study demonstrates that curcumin induces differentiation of stem cells as evaluated by significant increase in the gene expression of cardiac specific transcription factor and cardiac specific proteins. We were further interested in evaluating the combined effect of NO and curcumin in stem cell differentiation. Our results demonstrate enhanced differentiation of stem cells into cells of mixed lineage with combination of NO donor and curcumin possibly due to increased expression of endothelial NOS (NOS-3). Previous studies have shown that curcumin mediates significant relaxant effect in isolated porcine coronary arteries in both endothelial intact and endothelial denuded arteries in a concentration dependent manner and inhibitor of NO synthesis (L-NNA), methylene blue (sGC inhibitor) or propranolol significantly decreased the relaxation caused by curcumin (Xue et al., 2007). The study further concludes that relaxation of porcine arterial rings by curcumin is mediated through NO, cGMP and β-adrenoreceptors (Xu et al., 2007). Curcumin has been shown to affect number of molecular targets including transcription factor p53 and cyclin-dependent kinase inhibitor (CDKI) p21 via its role as a pro- and antioxidant (Sandur et al., 2007). Our studies indicate that combination of curcumin + NOC-18 increased the accumulation of transcription factor p53 with concomitant increase in the protein levels of CDKI p21 and cardiac specific protein MLC2 and troponin I compared to either of the two agents alone. These studies suggest that although polyphenol curcumin alone can induce the differentiation of ES cells combination of NOC-18 and curcumin further enhanced the differentiation of stem cells. Previous studies have shown that p53 induces differentiation of stem cells by inhibiting the gene expression of nanog, a stem cell marker (Yang, 2005; Lin et al., 2005). We recently demonstrated that cGMP is important for the processes involved in differentiation of human embryonic stem cells (Mujoo et al., 2008). The role of cAMP in differentiation of stem cells towards various lineages has been established many years ago (Goldstein et al., 1990). However, it is not clear whether curcumin can influence differentiation of human ES cells via a mechanism associated with cAMP or cGMP. It is also not known whether curcumin is able to influence steady state baseline levels of cyclic nucleotides. Our results indicate that long-term presence of curcumin in the partially differentiated cultures of stem cells substantially increases the rate of cAMP and cGMP degradation in the extracts. However, curcumin added directly to the extracts showed an opposite effect and significantly suppressed cAMP and cGMP. This might be due to direct suppression of some cyclic nucleotide-degrading enzyme (phosphodiesterase) by curcumin which may lead to a feedback increase in the rate of cyclic nucleotide degradation consistent with observed changes in baseline cAMP levels. However, elevated baseline cGMP levels in our studies might suggest that some factors contributing to synthesis of cGMP are up regulated during prolonged exposure to curcumin (NO-synthase, soluble or particulate guanylyl cyclases). Future studies will focus on evaluating the expression and activity of cAMP and GMP dependent protein kinase(s) (PKA & PKG) and phospodiesterases in response to curcumin to completely evaluate the role of NO signaling in further enhancing the differentiation induced by curcumin.

MATERIALS AND METHODS

Reagents and antibodies

Curcumin was purchased from Sigma Chemical Co and prepared in DMSO as a 10 mmol/L stock and stored at -80°C. NOC-18 (DETA-NO, slow release NO donor ($t_{1/2}$ 24 hours)), BAY 41-2272 (3-(4-amino-5-cyclopropylpyrimidin-2-yl)-1-(2-fluorobenzyl)-1*H*-pyrazolo[3,4-*b*]pyridine), YC-1(3-(5′-Hydroxymethyl-2′-furyl)-1-benzyl indazole, IBMX (3-isobutyl-1-methyl xanthine) and cGMP analog (8-bromo-cGMP) were purchased from Calbiochem, San Diego, CA. Monoclonal anti-β-actin (AC-15) antibody was obtained from Sigma Chemical Co, St. Louis, MO. Anti-MLC2, anti-smooth muscle actin, anti-p53 (DO-1) and phospho ERK1/2 antibodies were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Antibody against p21/WAF1/CIP1 was obtained from Millipore (Temecula, CA), antibody against ERK-1 from BD Transduction Laboratories, San Jose, CA, anti-eNOS and anti-troponin I antibodies were purchased from

Cell Signaling Technology, Beverly, MA.

Cell culture and differentiation of human ES cells

H-9 (WA-09, human embryonic stem cells; hESC) were purchased from WiCell Research Institute, Madison, WI and grown in 80% DMEM/F12, 20% knock-out serum replacer, 1 mmol/L L-glutamine, 0.1 mmol/L β-mercaptoethanol, 1 mmol non-essential amino acids supplemented with 4 ng/mL bFGF on mitotically-inactivated MEF feeder layers and matrigel. For random differentiation, the cells were dissociated using 2 mg/mL of collagenase IV (Invitrogen), washed and cultured in suspension in low attachment plates (Corning) in the differentiation medium containing 80% K/O-DMEM 1 mmol/L L-glutamine, 0.1 mmol/L β-mercaptoethanol, 1 mmol/L non-essential amino acids and 20% defined FBS (Hyclone). The media was changed on day 2 and day 4 and on day 6 the embryoid bodies (EBs) were transferred onto gelatin coated plates (3–4 EBs/cm^2) and cultured for additional days as described in the results. Cultures of undifferentiated ES cell were designated as day 0 control. ES cell- derived cardiomyocytes and other heterogeneous populations of the cells were analyzed by immunostaining, RT-PCR and Western blot.

Treatment of partially differentiated cells with curcumin and activators of NO-cGMP pathway

To examine the effect of curcumin and activators of NO-cGMP pathway in differentiation of H-9 cells, EBs (day 1 and day 2) and partially differentiated cells (day 7) were incubated with various concentrations of curcumin (5–20 μmol/L), NO donor-NOC-18 (5–10 μmol/L), allosteric sGC activators BAY 41-2272 (5 μmol/L) and YC-1 (5 μmol/L). The agents were added to the cell culture on day 1 and 2 or on day 7, 9, 11 and, 13 (multiple treatments). The cells were harvested between day 14–17 and marker genes and proteins were analyzed as indicated in the RESULTS section. Nitrate and nitrite concentrations were quantified by ion chromatography (ENO20 Analyzer; Eicom, Kyoto, Japan) (Bryan and Grisham., 2007).

Real-time RT-PCR

Total RNA from undifferentiated and differentiated cells was isolated using ultra Spec total RNA isolation reagent (Biotecx; Houston, TX) or TRIZOL reagent (Invitrogen, Carlsbad, CA). cDNA was prepared using high capacity cDNA archive kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. Real-time PCR assays for different subunits of sGC (α_1, β_1) various isoforms of NOSs, Nkx2.5, MLC2, cardiac troponin and GAPDH were purchased from Applied Biosystems and determined using the manufacturer's suggested protocol. All reactions were conducted using a 7900 HT Prizm Sequence Detection System for 40 cycles. The results were analyzed using the $_2{}^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen., 2001).

Western blot analysis

Cell cultures were washed with cold PBS and collected in cell extraction buffer (Invitrogen) supplemented with 1mmol/L PMSF and protease inhibitor cocktail (Sigma Chemical Company) for 30 min on ice. Equal protein aliquots were resolved on SDS-PAGE and transferred on to nitrocellulose membrane. The membranes were blocked for 45 min in a blocking buffer (5% milk in Tris buffered saline), washed and incubated with specific antibodies (p53 1:3000, p21 1:1000, MLC2 1:500, troponin I 1:1000, ERK 1/2 and total ERK-1 1:1000, eNOS 1:1000 and β-actin 1:3000–1:5000) for 2 h (most) or 30 min (β-actin) at room temperature. The membranes were exposed to ERK antibodies overnight at 4°C. Proteins were detected with HRP-conjugated secondary antibodies and visualized by enhanced chemiluminescence.

Immunostaining

H-9 cells grown in gelatin-coated chamber slides (partially differentiated cells) were exposed to vehicle control, curcumin (10 μmol/L) or turmeric (10 μmol/L may be contribute to 0.5–4 μmol/L of curcumin), NOC-18, combination of NOC-18 + curcumin or NOC-18 + turmeric for 24 h at 37°C. Slides were quickly washed with ice-cold PBS and fixed with 2% paraformaldehyde. Staining of primary antibodies was detected using Alexa-Fluor fluorescent-labeled secondary antibodies (Molecular Probes) and inverted fluorescent microscopy. Digital images were captured using Zeiss fluorescence microscope and processed with Zeiss imaging suit software.

Baseline cAMP and cGMP levels in differentiated human ES (H-9) cells

Differentiated human embryonic stem cells were harvested, washed 3 times with PBS and extracted with 0.2 mL of 0.1 mol/L HCl/well of 6-well plate. The extracts were centrifuged and cAMP and cGMP contents were assayed in the supernatant with kits from Cayman Chemical Co. Cell pellet was used to measure the protein and results were expressed in pmol cAMP or cGMP/mg protein. (Kots et al., 2008).

Degradation of cAMP and cGMP in the extracts of differentiated human ES (H-9) cells

Cells were washed 3 times with Dulbecco's PBS and lysed in ice-cold 50 mmol/L Triethanolamine-HCl, pH 7.6, containing 1 mmol/L EDTA and protease inhibitors (Calbiochem set V). Cells were briefly sonicated (10 sec at 50% power) and centrifuged at 3000 g for 5 min at 4°C to remove unbroken cells and nuclei, and the lysate was adjusted to 1.0–2.0 mg protein/mL concentration with lysis buffer. Samples containing 10–30 μg protein were incubated in the final volume of 20 μL in the presence of 5 mmol/L $MgCl_2$ and 10 μmol/L cAMP or cGMP for 15 min at 37°C. Reaction was stopped by boiling for 2 min and cAMP or cGMP contents were assayed with the corresponding kits from Cayman Chemical Co. Rate was calculated against control samples which were boiled prior to addition of cyclic nucleotides or contained lysis buffer instead of protein and expressed in pmol cAMP or cGMP degraded per min per mg protein. The assay was performed in the presence of 10 μmol/L curcumin or vehicle (0.1% DMSO).

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ABBREVIATIONS

cAMP, cyclic AMP; cGMP, cyclic GMP; CUR or Cur, curcumin; EB, embryoid bodies; ES cells, embryonic stem cells; hES, human embryonic stem cells; NO, nitric oxide; NOS, nitric oxide synthase; sGC, soluble guanylyl cyclase

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