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Chlorotyrosine promotes human aortic smooth muscle cell migration through increasing superoxide anion production and ERK1/2 activation

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Abstract

Chlorotyrosine is an oxidative product of hypochlorous acid and L-tyrosine, and is considered as a biomarker for oxidative stress and cardiovascular disease. However, it is not clear whether chlorotyrosine could directly contribute to vascular pathogenesis. In this study, we investigated the effect and potential mechanisms of chlorotyrosine on human aortic smooth muscle cell (AoSMC) migration. With Boyden chamber and wound healing assays, chlorotyrosine significantly increased AoSMC migration in a concentration- and time-dependent manner. In addition, chlorotyrosine significantly increased the expression of several key molecules related to cell migration including PDGF receptor-B (PDGFR-B), matrix metalloproteinases (MMP-1 and MMP-2) and integrins ($\alpha 3$, αV , and $\beta 3$) in AoSMC at both mRNA and protein levels. Furthermore, chlorotyrosine also increased superoxide anion generation in AoSMC with the fluorescent dye dihydroethidium (DHE) staining. Activation of mitogen-activated protein kinases (MAPKs) was analyzed with Bio-Plex Luminex immunoassay and western blotting. Chlorotyrosine induced a transient phosphorylation of ERK1/2, but not JNK and p38 MAPKs. Antioxidants including selenomethionine (SeMet) and Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) as well as ERK1/2 inhibitor PD98059 effectively blocked chlorotyrosine-induced AoSMC migration. Thus, these findings demonstrate new biological functions of chlorotyrosine in human SMC migration, which may play a crucial role in the vascular lesion formation.

Keywords

Chlorotyrosine; L-tyrosine; smooth muscle cell migration; PDGFR-B; MMPs; integrin; superoxide anion; ERK1/2

1. Introduction

Oxidative modification of proteins or amino acids is believed to be one of important pathogenic factors for many diseases including atherosclerosis [1]. Myeloperoxidase (MPO), a major phagocyte secreted protein, has been implicated in oxidative damage at sites of inflammation. It catalyzes the two-electron peroxidation of chloride to generate hypochlorous acid (HOCl) [2], a potent chlorinating oxidant, capable of chlorinating

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tyrosine into chlorotyrosine. Levels of chlorotyrosine are increased in atherosclerotic tissues [3] and the blood of in patients with inflammatory conditions including atherosclerosis [4,25,26]. The chlorination of apolipoprotein A-I was found to impair its cardioprotective ability to remove excess cellular cholesterol from lipid-laden macrophages in the artery wall [5]. Furthermore, increased chlorotyrosine was observed in the neointimal hyperplasia following vascular injury [6]. Chlorotyrosine was considered as a biomarker for coronary artery disease. However, biological functions of chlorotyrosine in the vascular system are largely unknown.

Vascular smooth muscle cells (VSMC) in the tunica media can acquire the ability to proliferate, migrate, and accumulate within the intimal layer of arteries under the influence of chemotaxis-inducing chemokines, cytokines and growth factors [7]. Migration and proliferation of VSMC are the key process of neointimal hyperplasia and atherosclerosis progression [7]. Many growth factors such as platelet-derived growth factor-BB (PDGF-BB) play crucial roles in VSMC migration. Matrix metalloproteinases (MMPs) and integrins provide permissive effects for VSMC migration by breaking down major extracellular barriers such as basal membranes, interstitial collagens, and proteoglycans [8].

Reactive oxygen species (ROS) including superoxide anion are generated by a variety of extracellular and intracellular mechanisms and are involved in several signal transduction pathways such as mitogen-activated protein kinases (MAPKs) [9]. ROS mediate cellular signaling pathways in VSMC proliferation and migration associated with atherosclerosis [10]. Thus, increased ROS generation may contribute to cardiovascular diseases such as atherosclerosis, angina pectoris, and myocardial infarction [11]. Antioxidants are believed to reduce the incidence of coronary artery disease [12]. Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) is a cell permeable superoxide dismutase (SOD) mimetic, which is an effective superoxide anion inhibitor [13]. Seleno-L-methionine (SeMet) is an effective antioxidant by increasing the activity of glutathione peroxidase [14].

We hypothesized that chlorotyrosine may have directly effects on the vascular system. In the current study, we investigated the effect and potential mechanisms of chlorotyrosine on the migration of human aortic smooth muscle cells (AoSMC). The expression of PDGFR-B, MMPs and integrins were studied at mRNA and protein levels in chlorotyrosine-treated cells. We further investigated the involvement of superoxide anion generation and MAPK activation in chlorotyrosine-induced AoSMC migration. This study provides experimental evidence of biologic functions of chlorotyrosine in human VSMC, which may contribute to the vascular lesion formation.

2. Methods

2.1. Chemicals and reagents

L-Tyrosine, 3-chloro-L-tyrosine (MW 215.64), anti-human β -actin antibody and SeMet were obtained from Sigma-Aldrich (St. Louis, MO). Anti-human integrin $\beta 3$ (CD61), integrin αV , and integrin $\alpha 3$ (CD49c) were purchased from BD Biosciences Pharmingen (San Diego, CA). Human platelet derived growth factor-BB (PDGF-BB), anti-human PDGFR-B antibody, and anti-human MMP1 were from R&D Systems, Inc. (Minneapolis, MN). Rabbit monoclonal antibodies specific for phosphor- and total ERK (p44/p42), JNK, and p38 were from Cell Signaling Technology, Inc. (Danvers, MA). Dihydroethidium (DHE) was obtained from Molecular Probes (Eugene, OR). MnTBAP was purchased from A.G. Scientific (San Diego, CA). Extracellular signal-regulated kinase (ERK) inhibitor (PD98059), JNK inhibitor (SB203580), p38 inhibitor (SP600125), and calcein-AM were obtained from Calbiochem Inc. (San Diego, CA). Trizol reagent was from Invitrogen (Carlsbad, CA).

2.2. Cell culture

AoSMC were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). The cells were routinely cultured in Smooth Muscle Medium-2 (SmGM-2) with growth factors and antibiotic (SmGM-2 Bullet Kit) supplemented with 10% fetal calf serum (FCS). Prior to each experiment, AoSMC were placed in the SmGM-2 medium with 1% FCS, without addition of growth factors (basal medium) for 16 h (serum starvation). AoSMC were used between passages 3 and 7.

2.3. Cell migration assay

Cell migration was measured with a modified Boyden chamber assay including Fluoroblok transwell migration plates as described in our previous study [15]. Briefly, serum-starved cells were trypsin-harvested. Cell suspension in basal medium (250 μ l, 1×10^5 cells/well) seeded in the upper chamber (24-well plate). Then, 750 μ l of basal medium with chlorotyrosine or L-tyrosine was added to the lower chamber and incubated for 4 to 24 h. After incubation, the cells were labeled by 50 nM calcein-AM, a fluorescence dye. The fluorescence of the cells migrated to the lower chamber was measured from the bottom using a fluorescence microplate reader at 485/535 nm wavelength. Each treatment was repeated in 4 independent transwells. The migrated cells in the filters were also observed under fluorescence microscope (Olympus; Tokyo, Japan).

2.4. Wound healing assay

When AoSMC have grown to 90% confluence in the six-well plate, a scratch was made with a sterile cell scraper as described in our previous study [16]. The starting point was marked with a marker pen at the bottom of the plate. Cells were washed with basal medium twice and were incubated with chlorotyrosine (10^3 nM), L-tyrosine (10^3 nM), or PDGF-BB (10 ng/ml) in basal medium, and the cells were incubated for 16 h and stained with calcein-AM (50 nM). Photos were taken under fluorescence microscope.

2.5. RNA isolation and real time RT-PCR

Serum-starved AoSMC were treated with chlorotyrosine (10^3 nM) or L-tyrosine (10^3 nM) for 24 h, and total RNA was isolated for real time PCR analysis as described in our previous study [15]. Briefly, primers for human PDGFR-B, MMPs, integrins and β -actin were designed using Beacon Designer software (Table 1). Sample cycle threshold (Ct) values were determined from plots of relative fluorescence units (RFU) versus PCR cycle number during exponential amplification so that sample measurement comparisons were possible. The mRNA levels of these molecules in each sample were calculated as $2^{(40 - Ct)}$ and further normalized to β -actin expression as $[2^{(Ct \beta\text{-actin} - Ct \text{ gene of interest})}]$.

2.6. Flow cytometry analysis

Protein levels of integrin $\alpha 3$ and $\beta 3$ of AoSMC were determined with flow cytometry analysis as previously described [15]. Briefly, the cell suspension was immunoblocked in 10% human serum in 4°C for 20 min. Similar numbers of cells (1×10^6) were immunostained with manufacturer-recommended concentrations of antibodies: anti-integrin $\alpha 3$ and $\beta 3$ antibodies in 4°C for 30 min. Mouse IgG1 was used as an isotype control. PE-conjugated anti-mouse IgG1 was used as a secondary antibody. The cells were analyzed with FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). Data are presented as the percentage of positive cells corresponding to the mean fluorescence intensity in each experiment.

2.7. DHE staining

Cultured cells were treated with chlorotyrosine (10^3 nM) or L-tyrosine (10^3 nM) for 60 min. Superoxide anion production was determined with DHE staining. DHE is freely permeable to cells. In the presence of superoxide anion, DHE is oxidized to ethidium bromide (EtBr) with red fluorescence, and it is trapped by intercalating with DNA. EtBr is excited at 488 nm with an emission spectrum of 610 nm. Thus, the amount of EtBr detected with fluorescence measurement is well correlated to the level of cellular superoxide anion. One milliliter of DHE (3 μ M) in PBS was added into each well of six-well plates and incubated for 20 min at room temperature. DHE staining was analyzed with FACS Calibure flow cytometry.

2.8. MAPK activation

The MAPK phosphorylation state of AoSMC lysates was analyzed with Bio-Plex phosphoprotein and total target assays (Bio-Rad) as described in our previous publication [15]. Briefly, serum-starved AoSMC were treated with chlorotyrosine (10^3 nM) or L-tyrosine (10^3 nM), and the cell lysates were collected at different time points of 0, 5, 10, 20, 30, and 60 min. The phosphoprotein and total proteins of MAPKs were analyzed with a Luminex 100TM analyzer (Bio-Rad). Data are presented as the fold of phosphorylated to total MAPK proteins in each sample.

2.9. Western blot

AoSMC were treated with chlorotyrosine (10^3 nM) and L-tyrosine (10^3 nM) for 24 h, and the cell lysates were collected. Total cellular proteins were separated with 10% SDS-polyacrylamide gel electrophoresis and then transblotted onto the Hybond-P PVDF membrane (Amersham, Piscataway, NJ). After blocking the membrane with 5% nonfat dried milk, the membrane was probed with the respective primary antibodies overnight at 4°C against human integrin α V, MMP1, and PDGFR-B, respectively. The membranes were then incubated in the horseradish peroxidase (HRP)-linked secondary antibody for 50 min. The immunoreactive bands were detected with enhanced chemiluminescent (ECL) plus reagent kit. The band density was measured with the use of AlphaEaseFC 3.1.2 software (Alpha Innotech Corporation, San Leandro, CA) and normalized to β -actin expression.

The MAPK phosphorylation state of AoSMC lysates was also analyzed with western blotting. The cell lysates were collected as described as above. The phosphor- and total proteins of ERK1/2, JNK, and p38 were recognized with rabbit anti-human phospho- and total p44/42 MAPK (ERK), SAPK/JNK, and p38 antibodies. Followed with HRP-linked secondary antibody, the immunoreactive bands were detected with enhanced chemiluminescence (ECL) plus reagent kit.

2.10. MMP2 activity assay

The MMP2 activity of AoSMC was determined with the EnzoLyte™ 490 MMP-2 Assay Kit from AnaSpec Co. (San Jose, CA). Briefly, the supernatants of AoSMC cultures were collected and concentrated in YM-10 columns. One hundred microgram protein in 50 μ l of the concentrated supernatant was used for each assay. After activated by p-aminophenylmercuric acetate (APMA) for 15 min at 37°C, 50 μ l of a fluorogenic [an EDANS/DABCYL fluorescence resonance energy transfer (FRET) peptide] MMP2 substrate solution was added and the reaction was incubated for 30 min at room temperature. The fluorescence intensity representing the MMP2 activity was measured at 485/535 nm wavelength.

2.11. Statistical analysis

Data are presented as mean $\pm$ SEM in all experiments. Data from treated groups were compared with control groups with Student's t-test (two tailed). A value of $p < 0.05$ was considered significant.

3. Results

3.1. Chlorotyrosine promotes human AoSMC migration

In order to investigate the effect of chlorotyrosine on AoSMC migration, cells were treated for 24 h with increasing concentrations of chlorotyrosine (0-10⁴ nM) or L-tyrosine (10⁴ nM) in the Boyden chamber. The cells migrated through a polystyrene-membrane with 8 μ m-size pores were stained with calcein-AM, and measured with a fluorescence reader. As shown in Fig. 1A, L-tyrosine treatment had no effect on AoSMC migration compared with the no treatment group. However, we found that chlorotyrosine significantly ($p < 0.01$) increased AoSMC migration in a concentration-dependent manner (up to 149% compared with the L-tyrosine control). Chlorotyrosine at 10³ nM was found to reach the maximal effect on AoSMC migration. As a positive control, PDGF-BB significantly increased AoSMC migration up to 200%. The cells that had migrated to the lower chamber were also visualized under fluorescence microscope (Fig. 1B). To confirm the above findings, we performed a time course study at 4, 8, 16 and 24 h, and observed a significant effect of chlorotyrosine (10³ nM) on cell migration as early as 4 h after cell seeding (Fig. 1C).

To further confirm the effect of chlorotyrosine on AoSMC migration, we performed wound healing assay on single layered AoSMC and observed the cell morphology and migration behavior after treatment of chlorotyrosine (10³ nM) or L-tyrosine (10³ nM). As shown in Fig. 1D, chlorotyrosine substantially promoted cell migration during the wound healing process compared with the control.

3.2. Chlorotyrosine increases the expression of PDGFR-B, MMPs, and integrins in AoSMC

PDGF-BB, mediated through its receptor PDGFR-B, is a major regulator of SMC proliferation and migration. MMPs and integrins play an essential permissive role in cell migration. Expression of PDGFR-B, MMPs and integrins in AoSMC with chlorotyrosine and L-tyrosine treatment were evaluated with real-time PCR. As shown in Fig. 2, chlorotyrosine significantly ($p < 0.05$) increased the mRNA levels of PDGFR-B, integrin β 3, integrin α 3, integrin α V, MMP1 and MMP2 up to 187%, 237%, 137%, 163%, 200% and 200%, respectively, compared with the L-tyrosine treated controls.(100%).

Western blot and quantitation data further demonstrated that chlorotyrosine increased protein levels of PDGFR-B, integrins (β 3, α 3, and α V), and MMP1 expression up to 245%, 238%, 185%, 198%, and 217% respectively, compared with the L-tyrosine treatment (100%, Fig. 2B and C). Furthermore, chlorotyrosine significantly ($p < 0.01$) increased MMP2 activity in a concentration-dependent manner with the maximal effect up to 213% compared with the L-tyrosine treated control (Fig. 2D). Thus, these data demonstrate that chlorotyrosine upregulates several key molecules related to SMC migration including PDGFR-B, integrins, and MMPs at both mRNA and protein levels.

3.3. Superoxide anion production is involved in chlorotyrosine-induced AoSMC migration

ROS including superoxide anion mediate cellular signaling pathways in VSMC proliferation and migration associated with atherosclerosis (10). To explore whether superoxide anion could be involved in the molecular mechanism of chlorotyrosine-induced AoSMC migration, we examined the effects of chlorotyrosine on superoxide anion generation and antioxidant application. Superoxide anion levels were determined with fluorescence staining

with DHE, a compound chemically oxidized to the fluorescent DNA intercalating agent ethidium in proportion to the amount of superoxide present [6]. The fluorescence for superoxide anion was analyzed with flow cytometry analysis. As shown in Fig. 3A and B, chlorotyrosine (10^3 nM) significantly ($p < 0.01$) increased superoxide anion production up to 42% of cell population compared with L-tyrosine (10^3 nM) (26%) after 60 min treatment. These data indicate that chlorotyrosine can induce superoxide anion production, which might be involved in the signaling pathway of the chlorotyrosine-induced cell migration. Indeed, well characterized antioxidants including SeMet (20 μ M) and MnTBAP (3 μ M) were used in the AoSMC cultures with chlorotyrosine treatment, and effectively blocked chlorotyrosine-induced AoSMC migration (Fig. 3C and D). These data indicate that superoxide anion plays an important role in chlorotyrosine-induced AoSMC migration.

3.4. Chlorotyrosine activates ERK1/2, but not JNK or p38

Since the activation of MAPKs can be triggered by oxidative stress [9] and chlorotyrosine can increase superoxide anion production in this study, we further investigated whether chlorotyrosine could activate MAPKs. Major MAPKs include ERK1/2, and the c-Jun N-terminal protein kinase (JNK), and p38. Serum-starved AoSMC were incubated chlorotyrosine (10^3 nM) or L-tyrosine (10^3 nM), and the cell lysates were collected at different time points. A Bio-Plex assay kit (Bio-Rad bioscience) was used to detect phosphoproteins and total proteins of ERK2, JNK and p38. The ratio of phosphoproteins to total proteins at each time point was used to evaluate the phosphorylation levels of ERK2, JNK, and p38. As shown in Fig. 4A, chlorotyrosine (10^3 nM) substantially increased ERK2 phosphorylation with a peak up to 7-fold at 10 min, whereas L-tyrosine (10^3 nM) had very limited effects on ERK2 phosphorylation. This result was confirmed with western blot showed that increased phospho-ERK1/2 was detected from 5 to 20 minutes after chlorotyrosine treatment (Fig. 4B). However, chlorotyrosine had no significant effects on JNK and p38 phosphorylation (Fig. 4C, D, E, and F). These results suggest that chlorotyrosine can activate ERK1/2 signaling pathways on AoSMC.

To further test the impact of ERK1/2 activation on chlorotyrosine-induced AoSMC migration, we incubated AoSMC with an ERK1/2 specific inhibitor (PD98059, 10 μ M), a JNK inhibitor (SP600125, 10 μ M), or a p38 inhibitor (SB203580, 10 μ M) for 1 h prior and during chlorotyrosine or L-tyrosine treatment. Cell migration was evaluated with the modified Boyden chamber assay. As shown in Fig. 5A, ERK1/2 inhibitor PD98059 completely blocked the promoting effect of chlorotyrosine on AoSMC migration ($p < 0.01$). However, JNK and p38 inhibitors did not block chlorotyrosine-induced AoSMC migration (Fig. 5B). These findings suggest that chlorotyrosine promotes AoSMC migration through the ERK1/2 signaling pathway.

4. Discussion

Chlorotyrosine is considered as a MPO-specific oxidant marker, while it is not clear whether chlorotyrosine is directly involved in vascular pathogenesis. In the current study, we demonstrated that chlorotyrosine promotes AoSMC migration in a concentration- and time-dependent manner. In addition, chlorotyrosine increases the expression of several key molecules for AoSMC migration including PDGFR-B, MMPs and integrins in AoSMC. Furthermore, we have found that chlorotyrosine induces superoxide anion production in AoSMC and activates ERK1/2 signal pathway. These findings provide a direct link between chlorotyrosine and AoSMC migration.

Current investigation of the biological functions of free chlorotyrosine in vitro may have clinical significance. Free chlorotyrosine has been used as a biomarker for MPO-catalyzed oxidation damage in vivo and in vitro besides the protein-bound chlorotyrosine. For

example, human neutrophils convert free L-tyrosine to 3-chlorotyrosine by the MPO-hydrogen peroxide system using two independent models of phagocytosis [17]. This study suggests that chlorotyrosine generated by phagocytes may be involved in the oxidative and cytotoxic reactions at sites of inflammation and vascular disease. Free 3-chlorotyrosine levels are elevated in peritoneal inflammatory fluid from septic wild-type mice but not in the septic MPO-deficient mice [18]. Free 3-chlorotyrosine is the molecular fingerprint that implicates the MPO pathway in oxidative damage. In the current study, we have observed the direct effect of free 3-chlorotyrosine on AoSMC migration, which is the major mechanism of the vascular lesion formation. Therefore, we believe the current study is clinically relevant and increased levels of 3-chlorotyrosine under inflammation conditions may contribute to vascular disease.

In the current study, we have confirmed the specific effects of 3-chlorotyrosine on AoSMC migration with two negative controls (L-tyrosine and no treatment) and one positive control (PDGF-BB). L-tyrosine at the concentration equivalent to 3-chlorotyrosine had no effects on AoSMC migration, which was similar to the no treatment group. However, 3-chlorotyrosine significantly increased AoSMC migration compared to L-tyrosine or no treatment. Current study design is consistent with the other studies on chlorotyrosine which are always compared with tyrosine in either plasmas or tissues [19,20].

VSMC migration and proliferation play an important role in the vascular lesion formation including neointimal hyperplasia and atherosclerosis [7]. Many molecules are involved in this process. Integrins are a family of heterodimeric transmembrane glycoproteins consisting of noncovalently associated α and β chains [7]. The integrin complexes $\alpha V\beta 3$ and $\alpha V\beta 5$ have been shown to be expressed on VSMC and to regulate their migration through interactions with the extracellular matrix [21]. In the current study, we found that chlorotyrosine not only directly promotes SMC migration but also upregulates the expression of integrins (αV , $\beta 3$ and $\beta 5$), PDGFR-B, MMP1, and MMP2. These molecules could be responsible to the chlorotyrosine-induced AoSMC migration.

Oxidative stress is a state in which excess ROS overwhelms endogenous antioxidant systems. The effect of oxidative stress on endothelial cells and VSMC include direct oxidation of proteins and indirect modulation of kinase pathways such as PKC, ERK, Src, and Pho [9]. Many factors such as cytokine, drugs, and extracellular inflammation factors could increase ROS generation to regulate cellular reactions including cell proliferation and migration [10]. In the current study, we demonstrate that chlorotyrosine can increase the production of superoxide anion, a major ROS, in AoSMC after 60 min treatment. Functional significance of over production of superoxide anion is demonstrated by using antioxidants SeMet and MnTBAP, which completely blocked chlorotyrosine-induced AoSMC migration.

MAPK family members are key signaling molecules for many stimuli including regulation molecules and environmental factors, and ROS have been implicated in activation of ERK1/2 and p38 MAP kinase in VSMC [22]. MAPKs are activated by growth factors and other signaling molecules, and are critical in signaling pathways mediating cellular proliferation and migration [23]. Chlorotyrosine has been reported to be involved in ERK signaling pathway [24]. To further investigate the molecular mechanisms of chlorotyrosine-induced AoSMC migration, we determined the involvement of signal transduction pathways such as ERK1/2, p38, and JNK using both a classic western blotting and a new Bio-Plex Luminescence technology. We found that chlorotyrosine increased transient activation of ERK2, but not JNK and p38. In addition, a specific ERK1/2 inhibitor PD98059, but not JNK and p38 inhibitors, can completely inhibit the promoting effect of chlorotyrosine on AoSMC migration. It indicates that the activation of the ERK signaling pathway is essential for chlorotyrosine-induced AoSMC migration.

In conclusion, chlorotyrosine has direct effects on human AoSMC by promoting cell migration and upregulating the expression of PDGFR-B, integrins, and MMPs. These biological functions of chlorotyrosine may be mediated by increasing superoxide anion production and activating ERK1/2 signal pathway. Thus, current study provides direct evidence that chlorotyrosine is a biologic active molecule, which may contribute to the vascular lesion formation.

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Fig. 1.

Effect of chlorotyrosine on AoSMC migration. **A.** Concentration-dependent study. AoSMC were seeded onto the transwell plate with chlorotyrosine (0–10⁴ nM) or L-tyrosine for 24 h. AoSMC migrating was analyzed with a fluorescence calcein-AM staining and quantitation. Results are expressed as percentage of the control. **B.** The representative photomicrographs of the migrated cells to the lower chambers after calcein-AM staining. **a.** L-tyrosine; **b.** chlorotyrosine (10³ nM); and **c.** PDGF-BB (10 ng/ml) as a positive control. **C.** Time course study. AoSMC were seeded onto the transwell plate with 10³ nM chlorotyrosine or L-tyrosine. AoSMC migration was studied at 4, 8, 16, and 24 h. Each bar represents the mean $\pm$ SEM. * p < 0.05 and ** p < 0.01 compared with the control (L-tyrosine). n = 4 per group. **D.** The representative photomicrographs of wound healing assay. Confluent AoSMC were wounded by a scratch injury line made with a sterile cell scraper. Cells were then treated with L-tyrosine (10³ nM); chlorotyrosine (10³ nM); or PDGF-BB (10 ng/ml). Photos were taken at 16 h. Dotted white lines delimit the initially wounded regions. **a.** L-tyrosine (10³ nM); **b.** chlorotyrosine (10³ nM); and **c.** PDGF-BB (10 ng/ml) as a positive control.

Fig. 2.

Effects of chlorotyrosine on the expression of PDGFR-B, integrins ($\beta 3$, $\alpha 3$, and αV), and MMP1 as well as MMP2 activity in AoSMC. Cells were treated with chlorotyrosine (10^3 nM) or L-tyrosine (10^3 nM) for 24 h. The cells were harvested and cell lysates were prepared. **A.** The mRNA levels of PDGFR-B, MMP1, MMP2, integrin $\alpha 3$, integrin αV , and integrin $\beta 3$ were analyzed with real-time PCR and were normalized to the housekeeping gene β -actin as [$2^{-(Ct \beta\text{-actin} - Ct \text{ gene of interest})}$]. **B.** Equal amount of 15 μ g cell lysate proteins were used to run western blot against human PDGFR-B, integrins ($\beta 3$, $\alpha 3$, and αV), and MMP1. **C.** Quantitation of the western blot protein bands. The band density was measured with an AlphaEaseFC 3.1.2 software and normalized to β -actin levels. **D.** MMP2 activity. The culture supernatants were collected and concentrated. The supernatant was treated with APMA for 15 min at 37°C, and 50 μ l of a fluorogenic FRET conjugated MMP2 substrate solution was added and incubated for 30 min. The fluorescence intensity representing the MMP2 activity was measured at 485/535 nm wavelength. Results are expressed as percentage of controls (L-tyrosine). * $p < 0.05$ and ** $p < 0.01$, $n = 3$. Data represent as mean $\pm$ SEM.

Fig. 3.

Roles of superoxide anion in chlorotyrosine-induced AoSMC migration. The cells treated with chlorotyrosine (10^3 nM) or L-tyrosine (10^3 nM) for 60 min were stained with DHE and analyzed with flow cytometry. **A.** Representative histograms of DHE staining and flow cytometry analysis. **B.** Quantitation of DHE staining and flow cytometry analysis. **C.** and **D.**, Effect of antioxidant SeMet and MnTBAP on chlorotyrosine-induced AoSMC migration with the transwell system. AoSMC were treated with chlorotyrosine (10^3 nM) or L-tyrosine (10^3 nM), in the presence or absence of SeMet (20 μ M) or MnTBAP (3 μ M) for 24 h. AoSMC migrated to the lower chamber were measured with calcein-AM staining and quantitation. Results are expressed as percentage of controls (L-tyrosine). Each bar represents the mean $\pm$ SEM. * p < 0.05 and ** p < 0.01 compared with the control (L-tyrosine). n = 4 per group.

Fig. 4. Role of MAPK in chlorotyrosine-induced AoSMC migration. **A**, **C**, and **E**. Bio-Plex Luminex immunoassay of phosphorylated and total ERK2, JNK, and p38 proteins in chlorotyrosine (10^3 nM) and L-tyrosine (10^3 nM) treated AoSMC lysates at different time points. Data are presented as the fold increase of the ratio of phosphorelated and total ERK2, JNK, and p38 proteins versus the untreated samples (0 min time point). $n = 3$ per group. **B**, **D**, and **F**. Western blot of phosphorylated and total ERK2, JNK, and p38 proteins in chlorotyrosine (10^3 nM) treated AoSMC lysates at different time points.

Fig. 5.

Effects of the inhibitors of ERK1/2, JNK and p38 on chlorotyrosine-induced AoSMC migration. AoSMC were treated with chlorotyrosine (10^3 nM) or L-tyrosine (10^3 nM), in the presence or absence of (A) ERK1/2 inhibitor (PD98059, 10 μ M), (B) JNK inhibitor (SP600125, 10 μ M) or p38 inhibitor (SB203580, 10 μ M) for 24 h. Results are expressed as percentage of controls (L-tyrosine). AoSMC migration was determined with the transwell system. Each bar represents the mean $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ compared with the control (L-tyrosine). $n = 4$ per group.

Table 1

Sequence details of individual pairs of primers

Gene	Gene Bank No.	Forward Primer	Reverse Primer
PDGFR-B	NM_002609	CCTTACCACATCCGCTCCATCC	CATTCACACTCTCCGTCACATTGC
MMP-2	AY738117	CAACTACAACCTTCTCCCTCGCA	GGTCACATCGCTCCAGACTTG
MMP-1	NM_002421	TCACAGCTTCCCAGCGACTC	TCAACTTGCCTCCCATCATTCTTC
Integrin α v	NM_002210	GATTTCTTCGTGCCCAGCG	GCGGGTAGAAGACCAGTCAC
Integrin β 3	NM_000212	ATGCTTCATCCATCATCGGTGTC	GGTCACCTGGTCAGTTAGCGT
Integrin β 5	NM_002213	ACCTATGTCTGCGGCCTGTG	TTCTGGTACACGCTCTGGTTCT
Integrin α 3	NM_005501	CTAGAGTGCCCCATCCCTGAT	TGTAATCCTCGATGAAGGTGCTG
β -actin	BC013835	CTGGAACGGTGAAGGTGACA	GGAAGCACTTCCTGGACTTGAT