naling cascades coupling to the ligand binding of the AT_4 receptors are not clear and may involve multiple signaling pathways [14]. Recent evidence also suggests that an activation of AT_1 receptors could be involved in the cellular effects of Ang IV on intracellular signaling events [17, 18]. At the moment, the expression of the AT_4 receptor in the carotid body and its regulation by hypoxia remains unknown. In this study, we tested the hypothesis that AT_4 receptors are present in the rat carotid body and play a role in the adaptive change in chronic hypoxia. In addition, we investigated the effect of Ang IV on the $[Ca^{2+}]_i$ level of the glomus cells because of the physiological significance of the $[Ca^{2+}]_i$ elevation in the chemotransduction.

MATERIALS AND METHODOLOGY

Preparation of Animals

The experimental protocol for this study was approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong and the Animal Ethical Committee of the Chinese University of Hong Kong. For the exposure of rats in normobaric hypoxia [19], one-month-old Sprague-Dawley rats were placed in a chamber made of Perspex. The chamber was continuously vented by pumping in room air (2 liter/min) and the oxygen fraction of the gas inside the chamber was kept at $10\pm0.5\%$. The oxygen level was continuously monitored by an oxygen analyser (Vacumed, CA, USA) which provided the servo-feedback signal for the control of solenoid values which gated the inflow of pure nitrogen. Animals were kept in the chamber for 4-5 weeks and they were freely accessible to water and chow. The humidity and carbon dioxide level was maintained by dehumidifier and soda lime. In every 2-3 days, the chamber was opened for 30 min for regular maintenance. For normoxic controls, litters were kept in the same room but were supplied with room air.

Isolation of Carotid Body and Dissociation of Type-I Glomus Cells

Following deep anesthesia with halothane, rats were decapitated and the carotid bifurcation was excised rapidly. For the dissociation of glomus cells, the carotid body was carefully dissected free from the bifurcation in chilled rat Ringer oxygenated with 95% O_2 and 5% CO_2. The carotid body was then incubated in a tissue bath with collagenase (0.06%) and protease (0.02%) in oxygenated Ringer for 30 min at 35 ± 1°C [8]. Following enzymatic treatment, the carotid body cells were dispersed by gently triturating with glass pipettes. Cells were incubated in 5 μM fura-2 acetoxymethyl ester (fura-2AM, Molecular Probes) for 30 min at room temperature. The cells were then centrifuged at 200 g for 5 min and prepared for the spectrofluorimetric measurement of $[Ca^{2+}]_i$. Glomus cells in clusters of 8-10 cells were studied and the morphological criteria for their confirmation as glomus cells was according to the methods used in previous reports [8, 19].

Fluorescence-Labeled Binding Assay

Carotid bifurcations were excised from normoxic and hypoxic rats (n=8) under deep anesthesia. The bifurcations were rinsed in PBS and frozen in iso-pentane. Cryosections (8 μm) were cut on a Cryostat and fixed with freshly prepared 4% paraformaldehyde (PFA) for 30 min. Sections were washed with PBS at room temperature for 10 min and then fixed in 2% PFA for 10 min, followed by PBS rinse for 10 min. After air dry, sections were incubated for 30 min at room temperature with one of the following conditions, which were described in details in a previous report [20]: (1) 20 nM fluorescein isothiocyanate (FITC)-labeled Ang IV (Auspep, Australia) in 5 ml Ang IV incubation buffer, which contained 59.3 μl of 50 μM plummers inhibitor (DL-2-2-Mercaptomethyl-3-guanidinoetyhiopropanoic acid (Calbiochem, CA, USA)) and 17.2 μl of 11 μM bestatin and 30 mg of 0.6% BSA in PBS; (2) pre-incubation with 10 μM non-labeled Ang IV (angiotensin II (3-8) (Auspep, Australia)) for 15 min followed by 5 mL Ang IV incubation buffer with 20 nM FITC-labeled Ang IV; (3) 20 nM fluorescein isothiocyanate (FITC)-labeled Ang IV in 5 ml Ang IV incubation buffer with 4.8 μl of 1mg/ml (1 μM) Sarile ([Sar^1, Ile^8]-angiotensin II; Sar-Arg-Val-Tyr-Ile-His-Pro-Ile). Following incubation, slides were washed twice in PBS for 1 min and then by double distilled water for 30 min at 4°C. Sections were dry overnight and examined with a fluorescent microscope with a DC 200 digital camera (Leica Microsystems Ltd., Heerbrugg, Switzerland). Non-specific bindings were assessed in sections with the non-labeled Ang IV and the specific bindings of Ang IV were demonstrated in sections with Sarile that is not readily metabolized to Ang III and Ang IV.

Immunohistochemistry

Cryosections were then processed for indirect immunofluorescent double staining as reported previously [7]. Briefly, sections were incubated overnight at 4°C with rabbit anti-rat AT_4 receptor serum, diluted to 1:1000, followed with anti-tyrosine hydroxylase serum (Chemicon International, USA), diluted to 1:300 overnight at 4°C. After rinses in PBS, the primary antibodies were detected using anti-rabbit serum labeled with rhodamine (1:100, Jackson, USA) for AT_4 receptor and anti-sheep serum labeled with AMCA (1:100, Jackson, USA) for TH at room temperature for 2 h. Positive immunoreactivity for AT_4 receptor (red) and for TH (blue) was examined with a fluorescent microscope with a DC 200 digital camera (Leica Microsystems Ltd., Heerbrugg, Switzerland). For the control, primary antibodies were substituted with buffer and sections were incubated with rabbit nonimmune serum.

Western Blot Analysis

The procedures were reported previously [7]. Carotid bodies were dissected free and frozen at -70°C. Carotid bodies (n=20) were homogenized at 4°C in water (1:9, w/v) containing 10 mM EDTA and 1 mM phenylmethylsulphonyl fluoride. The protein from resultant supernatant was determined (Bio-Rad Protein Assay) for sodium dodecy sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins (5-10 mg/lane) were subjected to electrophoresis on a 12% (w/v) polyacrylamide gel in SDS, and the gel was subsequently processed for electroblotting to polyvinylidene difluoride (PVDF) membrane. The blotted PVDF membrane was saturated with 5% (w/v) of skimmed milk in PBS, pH 7.4 and 0.1% (w/v) of Tween 20 for 1 h at room temperature. The membrane was sequentially incubated in rabbit anti-rat AT_4 receptor serum (1:2500) overnight at 4°C and a horse-