APPLICATION OF THE HMOX1 PROMOTER/ENHANCER ASSAY FOR ROUTINE DETERMINATION OF CELLULAR STRESS INDUCED BY CIGARETTE SMOKE

Constanze Knörr-Wittmann*, Stephan Gebel, Thomas Müller; PHILIP MORRIS Research Laboratories GmbH, Cologne, Germany; *e-mail: Constanze.Knoerr@pmintl.com

Introduction

Cigarette smoke (CS) harbors a strong oxidative stress potential, which broadly impacts exposed cells. A special feature of the CS-provoked cellular stress response is the strong transcriptional up-regulation of phase II and antioxidant-related genes, among which the expression of the gene encoding heme oxygenase-1 (hmx1) is most prominent. This has been seen in vitro and in vivo cDNA microarray studies [1, 2], suggesting that HO-1 plays a central role in the cellular defense mechanisms against oxidative stress. HO-1 catalyzes the rate-limiting reaction in the catabolism of heme, yielding products with both antioxidant and cytoprotective activities. Based on these characteristics, as well as on the strong transcriptional inducibility under various unrelated stress conditions, the expression of hmx1 is considered a reliable marker of cellular (oxidative) stress.

We previously presented first results of the hmx1 promoter/enhancer assay [3], demonstrating that a hmx1 promoter/enhancer reporter (luciferase) gene construct stably transfected into NIH3T3 cells is responsive to cigarette smoke fractions and single compounds at subtoxic concentrations. Here we present an optimized protocol for a higher (96-well format vs single petri dish) and faster (1 day vs 3 days) sample throughput.

Results

1. hmx1 Promoter/Enhancer Region

Fig. 1: The 15kb hmx1 promoter/enhancer region fused to the firefly luciferase gene was used to generate the transgenic cell line NIH3T3/pHO15luc.

2. Biological Significance

Fig. 2: CS-dependent endogenous hmx1 and ectopic luciferase gene expression in NIH3T3/pHO15luc cells are regulated in a similar way.

3. hmx1 Promoter/Enhancer Assay Design

Fig. 3: Effects of GVP and TPM on luciferase activity at different concentrations. Equal GVP and TPM concentrations are calculated on a puff basis.

4. First Application

Fig. 4: Concentration-dependent activity of luciferase activity by CdCl2 (used as positive control); two independent experiments on one day.

Summary

The hmx1 promoter/enhancer in vitro assay has been optimized for routine determination of cellular stress using cigarette smoke fractions and single compounds. • Luciferase expression reflects mild-type induction of hmx1. • 23 test samples in quadruplicate can be analyzed and compared on one plate. • The assay can be performed in a 96-well format within one day. • Intraday and interday variability were very low indicating that the assay would be useful for measuring cellular (oxidative) stress.

Materials and Methods

The transgenic cell line NIH3T3/pHO15luc was created by transfection of NIH3T3 cells with the plasmids pH015luc (kindly provided by Dr. J. Albrecht) containing the 15 kb promoter/enhancer region of the murine hmx1 gene fused to the firefly luciferase gene (Fig. 1). NIH3T3/pHO15luc cells in culture were kept under standard conditions with 400 mg/ml zeocin. For testing various concentrations of CS fractions or single compounds in quadruplicate, cells were seeded in black 96-well plates, starved (24 hours, 0.5% FCS) and then exposed in DMEM 0.5% FCS. After 7 hours WST-1 Cell Proliferation Reagent (Roche, Germany) was added to each well and cell proliferation/viability was determined. After a washing step, luciferase activity was measured directly in each well with the Luciferase Reporter Assay Kit (Roche, Germany). Chemiluminescence was determined and quantified by the luminometer “Fluostar Optima BMG” (BMG Labtechnologies, Germany). Results from the WST-1 Assay were used for normalization of each well on the basis of viable cell number. Luciferase activity was expressed as Fold Induction of treated to control samples. SD (standard deviation) was determined for Fold Induction. Wells showing cytotoxic effects, as detected by the WST-1 assay, were not used for analysis.

Cigarette Smoke Fractions: Smoke-bubbling PBS (PBSR) was prepared by bubbling 30 puffs of mainstream CS from the Standard Reference Cigarette 2R1 through 16 ml PBS-Dulbecco. Gas/particulate phase (GVP) was prepared in the same way, but the CS was bubbled through 100 ml of PBS-Dulbecco (GVP, 0.045 puffs/ml 3 days). Total particulate matter (TPM) from three cigarettes (2R1 or 2MR) was collected on one glass fiber filter (44 mm diameter), determined gravimetrically, and then extracted with 15 ml dimethyl sulfoxide (DMSO).

Acknowledgement

We thank J. Albrecht (Oncogene-Discs Foundation, New Orleans) for providing us with the plasmid pH015luc and G. Schröder for adlibitotechnical assistance.

References
