Serum and Extracellular Calcium Modulate Induction of Cytochrome P-450IA1 in Human Keratinocytes*

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Culture conditions allowing for cytochrome P-450IA1 induction by 2,3,7,8-tetrachlorodibenzofuran (TCDF) in normal human keratinocytes (HK) were investigated. HK grown in serum-free low extracellular Ca²⁺ (0.1 mM) medium did not accumulate P-450IA1 mRNA in response to TCDF. If, however, the cultures were pretreated for more than 24 h with either serum or elevated extracellular Ca²⁺ (2.0 mM), induction of P-450IA1 was obtained by TCDF. Serum and elevated Ca²⁺ concentrations were found to be additive in this respect. When analyzing HK derived from five individuals, no apparent difference was found in the relative induction of P-450IA1 mRNA in response to TCDF. If, however, the elevated Ca²⁺ could be conferred to a reporter gene by the corresponding to the differentiation specific enzyme epidermal transglutaminase. This finding, together with the known differentiation promoting effects of serum and elevated Ca²⁺, suggest that terminal differentiation is necessary for P-450IA1 induction in HK by Ah receptor ligands.

Epidermal cells offer a first defense as they exhibit various detoxifying enzyme activities against potentially harmful molecules in the environment. Polycyclic aromatic hydrocarbons (PAH) such as 3-methylcholantrene and benz(a)anthracene have been shown to induce aryl hydrocarbon (AH) hydroxylase activity in both the epidermis and in isolated keratinocytes in vitro (1–3). Although AH hydroxylase is involved in oxidative detoxification, metabolism via this enzyme can also result in the activation of xenobiotics to toxic, mutagenic, and carcinogenic species (4). The major fraction of PAH-induced AH hydroxylase activity in rodent skin has been attributed to the Cyp2IA1 gene product, the microsomal cytochrome P-450IA1 (3).

Polychlorinated PAHs, such as the dioxins, dibenzofurans and biphenyls, are the most potent inducers of P-450IA1 and bind the ubiquitously distributed low abundance cytosolic receptor protein (the Ah or dioxin receptor) with the highest affinity (5). The molecular mechanism of Ah receptor function has been delineated using cells of hepatic origin and is analogous to steroid receptors as it involves ligand binding, activation (including loss of the 90-kDa heat shock protein), nuclear translocation, and subsequent binding to specific DNA sequences which could lead to enhanced transcription of specific genes (6). Multiple copies of the well characterized binding motif for the liganded Ah receptor are located upstream of the rat and mouse CypIA1 genes (7, 8). The ~1140 to +2435 region of the human CYPIA1 has been shown to confer inducibility by PAHs via at least two regulatory elements to a reporter gene when transfected to a human epidermis derived cell line (9).

The Ah receptor is a necessary, although not always sufficient, mediator of the pleiotrophic biological response elicited by polychlorinated PAHs (Ref. 10 and references therein). Generally, large species variations exist in the response that include epithelial hyperplasia, thymic involution, liver damage, and teratogenic- and tumor-promoting effects. The most well established effect of polychlorinated PAHs in humans is a hyperkeratotic and metaplastic response of the hair follicles and interfollicular epidermis, leading to persistent acne-like lesions called chloracne (11).

In this study we have investigated the culture requirements for the induced expression of P-450IA1 by 2,3,7,8-tetrachlorodibenzofuran in normal human keratinocytes (HK). The advantages of using HK compared with transformed cell lines are the possibility to induce differentiation and the similarity in the degree of differentiation between cell cultures established from different donors. We show that under in vitro conditions that select for an undifferentiated basal-like phenotype, TCDF cannot induce P-450IA1 expression unless differentiation is allowed by raising the extracellular Ca²⁺ concentration and/or by treatment with serum.

MATERIALS AND METHODS

Cell Isolation and Cultivation—All chemicals, medium, and growth factors were purchased from Sigma, unless stated otherwise. HK were isolated from adult or newborn donors as described previously (12), with the modifications that the epidermis was separated from dermis by treatment with 0.5% Pronase for 20–40 min at 37 °C and that protease digestion was stopped by washing cells in MCDB 153 with 1% bovine serum albumin. Cells were seeded at clonal density onto a layer of irradiated (>30 gray) Swiss 3T3 fibroblasts. In most experiments, cells were grown in MCDB 153 medium, supplemented with 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 0.4 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 100 μg/ml bovine pituitary extract (prepared as described in Ref. 13, bovine pituitaries purchased from Pel-Freeze), 100 μg/ml streptomycin, 100 IU/ml penicillin (GIBCO), and 0.25 μg/ml fungizone (GIBCO) until near confluence when they were subcultured and plated onto fibro-
necin and collagen I-coated cell-culture plastic (14). The Ca\textsuperscript{2+} concentration of the MCDB 153 medium was adjusted to 0.1 mM (referred to as "low Ca\textsuperscript{2+}"). The "high Ca\textsuperscript{2+}") concentration used was 2.0 mM and "serum" refers to substitution of bovine pituitary extract with fetal calf serum (FCS, Hyclone) that had been depleted of divalent cations (15). In the dose-response and time course studies (Fig. 1) cells were grown in Dulbecco's modified Eagle's medium; Ham's F-12 3:1 medium (GIBCO) supplemented with the above-mentioned concentrations of epidermal growth factor, insulin, hydrocortisone, penicillin, streptomycin, fungizone, and 5% FCS. Cultures were treated with TCDF (Cambridge Isotope Laboratories) dissolved in dimethyl sulfoxide (Me\textsubscript{2}SO), whereas control cultures received solvent only, not exceeding a final concentration of 0.1%. Each experiment was performed at least twice using cells derived from different donors. No effect of TCDF on [\textsuperscript{3}H]thymidine uptake was detected under the culture conditions used. The HepG2 cell line was maintained in Dulbecco's modified Eagle's medium with 10% FCS.

**RNA Isolation and Northern Analysis**—Total RNA was isolated using acid-phenol extraction as described (16). RNA was fractionated through formaldehyde-agarose gels, blotted onto nylon membranes, then UV cross-linked, and filters were subsequently prehybridized, through formaldehyde-agarose gels, blotted onto nylon membranes, hybridized, washed according to standard procedures (17) prior to autoradiography. As a positive hybridization control, 4 pg/lane of total RNA was loaded in each lane. The blot was first probed for P-450IAl giving a signal at approximately 2.7 kilobases and then for GAPDH. 4 pg of total RNA was loaded in each lane. C, the time course of induction of P-450IAl mRNA by 50 nM TCDF for various lengths of time. 4 pg of total RNA was loaded in each lane.

**RESULTS**

Using culture conditions where a considerable portion of the cells showed a terminally differentiated phenotype, HK isolated from five individuals showed a comparable response with TCDF with regard to the increase in P-450IAl mRNA levels, giving EC\textsubscript{50} values of approximately 2 nM (Fig. 1, A and B). This concentration is in agreement to that found in other systems for induction of AH hydroxylase activity (21). The time course of induction showed that maximum steady-state levels of message were achieved by 24–48 h and that the mRNA remained high throughout the 72 h of the experiment (Fig. 1, C and D).

HK can be cultivated using serum-free and low extracellular Ca\textsuperscript{2+} conditions that suppress terminal differentiation and promote rapid proliferation resulting in a homogeneous basal-like population of cells. Under these culture conditions, TCDF treatment did not result in increased P-450IAl mRNA (Fig. 2 A lanes: low calcium). However, when differentiation was allowed by either pretreatment with elevated extracellular Ca\textsuperscript{2+} (lanes: high calcium) or serum (lanes: low calcium, serum), TCDF treatment resulted in increased P-450IAl mRNA levels. If the culture had received serum and high Ca\textsuperscript{2+} (lanes: high calcium, serum), there was an additive effect in that P-450IAl mRNA accumulated to very high levels when TCDF was present, representing at least a 30-fold induction compared with control cultures (basal levels are below the detection level). The relative induction by TCDF in serum or high Ca\textsuperscript{2+} compared with that in high Ca\textsuperscript{2+} plus serum varied to a certain degree between experiments. This is likely to be a consequence of variation in the confluency that will affect the differentiation state of HK in vitro (22). Induction was
Fig. 2. Inducibility of P-450IA1 mRNA levels by TCDF treatment under different culture conditions. Total RNA was prepared from preconfluent cultures of HK and HepG2 cells grown in either low Ca"+, low Ca"+ plus 10% serum, high Ca"+, or high Ca"+ plus serum for 48 h and then treated with 50 nM TCDF (TC) or Me6SO (C) for 4 h. A, shows a Northern blot analysis of P-450IA1 and GAPDH mRNA levels in HK. B, shows a Northern blot analysis of P-450IA1 and GAPDH mRNA levels in HepG2 cells. 10 µg of total RNA was loaded in each lane. The positive control is designated P (see "Material and Methods).

FIG. 3. Extracellular Ca"+ and serum concentration requirements for P-450IA1 mRNA accumulation after TCDF treatment. A, shows a Northern blot analysis of P-450IA1 and GAPDH mRNA levels in HK exposed to TCDF when grown in varying extracellular Ca"+ concentrations. The medium was changed to contain 5% serum and the indicated Ca"+ concentration 24 h before treatment with 50 nM TCDF or Me6SO for 4 h. 6 µg of total RNA was loaded in each lane. B, shows a Northern blot analysis of P-450IA1 and GAPDH mRNA levels in HepG2 cells. 10 µg of total RNA was loaded in each lane. The positive control is designated P.

FIG. 4. Time of pretreatment with high Ca"+ and serum required for P-450IA1 mRNA induction. Northern blot analysis of total RNA prepared from preconfluent HK cultures at different time points after the medium was changed to high Ca"+ plus 10% serum was shown. 50 nM TCDF (TC) or Me6SO (C) was included for the last 4 h of incubation, and all cultures were harvested at the same time point. 3 µg of total RNA was loaded in each lane and analyzed for P-450IA1 and GAPDH mRNA expression. The positive control is designated P.

FIG. 5. Expression of epidermal transglutaminase. A Northern analysis of transglutaminase (TG) and GAPDH mRNA in HK is shown. Total RNA was prepared from preconfluent HK grown in low Ca"+ and from HK that had received high Ca"+ plus serum for 24 h. 4 µg of total RNA was loaded in each lane.
shown in Fig. 6, the different pretreatments did potentiate treatment was analyzed in low Ca\textsuperscript{2+} and in cultures that had the effect of TCDF treatment. The highest level of CAT activity was seen in the absence of TCDF in high Ca\textsuperscript{2+} plus serum (as well as in high Ca\textsuperscript{2+} or serum) compared with the activity in TCDF (TC) or Me\textsubscript{3}SO (C). A, fold induction of CAT activity obtained in increased levels of P-450IAl mRNA in an undifferentiated population of normal HK and were interested to determine loss of response to TCDF and a reduction in the basal level of activity by high Ca\textsuperscript{2+} in murine keratinocytes (23), whereas serum has been shown to inhibit the growth of HK (25). We have shown that serum or high Ca\textsuperscript{2+} together had an additive effect. The serum and high Ca\textsuperscript{2+} treatment results in a responsive phenotype as a consequence of a changed gene program leading to differentiation as well as an allowance of P-450IA1 accumulation based on the following reasons: (i) immunohistochemically 3-methylcholanthrene-induced P-450IA1 has been localized to the suprabasal layers of rat skin (3) that contain cells that no longer proliferate but which are committed to terminal differentiation. This may be analogous to the herein described in vitro situation where the culture conditions allowing for P-450IA1 induction also lead to an increase in mRNA levels corresponding to the differentiation specific enzyme epidermal transglutaminase. (ii) A number of differentiation markers, such as keratin K1 and K10 and filaggrin, show enhanced expression at a restricted Ca\textsuperscript{2+} concentration of 0.10–0.16 mM in mouse keratinocytes (24). We found that P-450IA1 induction by TCDF occurred above a similar concentration, (0.19–0.21 mM Ca\textsuperscript{2+}), taking into account that HK are less sensitive to elevated extracellular Ca\textsuperscript{2+} than mouse keratinocytes (24, 28). (iii) The time required of serum and elevated Ca\textsuperscript{2+} before P-450IA1 mRNA induction is permitted suggest that a sequence of cellular changes have to occur. This would agree with a time requirement for elevated Ca\textsuperscript{2+} to act before enhanced metabolism of PAHs was obtained in murine keratinocytes using culture conditions with serum constantly present (29). In HK derived from five individuals we did not detect any major variations in the EC\textsubscript{50} values of TCDF with regard to induction of P-450IA1 mRNA. This system could however prove useful in studies addressing genetic variations in the human population that lead to differences in the response to Ah receptor ligands.

In addition to the species and the tissue, our results demonstrate that the cellular differentiation state is also an important modulator of the gene program altered by exposure to Ah receptor ligands. It will be important to define the limiting step/factor(s) that determine P-450IA1 induction by polychlorinated PAHs in HK. Interesting future questions are how molecular mechanisms regulating P-450IA1 expression correlate to changes in expression of other genes and biological effects in a cell type relevant to the human response to exposure.

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