Differential induction of *mafF*, *mafG* and *mafK* expression by electrophile-response-element activators

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The three small Maf proteins, MafF, MafG and MafK, have been implicated in a number of physiological processes, including development, differentiation, haematopoiesis and stress response. Here we report the constitutive expression of *mafF*, *mafG* and *mafK* in six human cell lines derived from various tissues (HepG2, IMR-32, K-562, HEK-293, RD and A549). The expression patterns of *mafF*, *mafG* and *mafK* varied widely among cell lines. Because small Maf proteins have been implicated in electrophile response element (EpRE)-mediated stress response, the ability of three EpRE activators [pyrrolidinedithiocarbamate (PDTC), phenylethyl isothiocyanate (PEITC) and t-butylhydroquinone (tBHQ)] to induce small Maf expression was examined in detail in HepG2 cells. Both PDTC and PEITC induced *mafF*, *mafG* and *mafK* expression, whereas tBHQ failed to markedly induce any of the three small Mafs. Where a response was observed,

mafF was induced to the greatest extent compared with *mafG* and *mafK*, and this response was transcriptionally mediated. PDTC also induced small Maf expression in the other cell lines examined, with patterns of induction varying among cell lines. The differences in expression among the cell lines examined, coupled with the induction patterns observed, indicate that the three small *maf* genes are stress-responsive, but may be regulated via differing mechanisms. Furthermore, the fact that tBHQ, PDTC and PEITC induce EpRE activity, but that tBHQ fails to markedly induce any of the small Mafs, suggests that up-regulation of small Mafs is not an absolute requirement for EpRE-mediated gene expression.

Key words: PDTC, PEITC, stress response, tBHQ, tissue-specific.

INTRODUCTION

The Maf basic leucine zipper (bZip) transcription factors have recently garnered much attention as potential players in a variety of physiological processes, including embryogenesis, development, haematopoiesis and stress response (reviewed in [1]). The Maf family is divided into two subclasses, large Mafs (vMaf, cMaf, MafB and Nrl) [2] and small Mafs (MafF, MafK [3] and MafG [4]). Both subclasses contain leucine-zipper motifs, which allow homodimerization as well as heterodimerization with a variety of other bZip proteins, including alternative members of the Maf family [5,5a], activator protein (AP)-1 factors [5,5a,6] and Cap 'N Collar (CNC) proteins (e.g. Nrf1, Nrf2, NF-E2 and p45) [7–10]. Recognition of and binding to specific DNA sequences by Maf-containing dimers is mediated, in large part, by a basic region with an adjacent extended homology region unique to Maf proteins [11]. The specific regulatory elements recognized by Maf-containing dimers vary somewhat, based on the identity of the dimerization partner present. Homodimers and interfamilial heterodimers bind Maf-recognition elements (MAREs) [6], whereas CNC–small Maf heterodimers recognize the NF-E2 *cis* element, which resembles a MARE half site linked to a TPA (PMA) response element (TRE) half site [12]. Large Mafs also contain an acidic transactivation domain absent in the small Maf proteins. Although they possess no inherent trans-activation activity, small Maf proteins can act as positive regulators of transcription by targeting transcriptionally active dimerization partners to specific DNA regulatory elements. Conversely, small Mafs can act as negative regulators of trans-

cription by recruiting transcriptional repressors or by forming homodimers that can then displace active dimers. Motohashi et al. [13] observed that the level of small Maf proteins affected both binding and transcriptional activity at a MARE (which also contained an NF-E2 site) in transgenic mice exhibiting varying degrees of MafK and/or MafG expression.

Recently, the involvement of Mafs in stress response has been the subject of much investigation. It has been suggested that CNC–Maf heterodimers mediate expression of stress-responsive genes, such as those involved in phase-II detoxification and GSH homeostasis by associating with the electrophile response element (EpRE; also known as the antioxidant response element or ARE) [14,15]. The EpRE consensus sequence [16–18] closely resembles the consensus sequence of the NF-E2 site, the recognition element for CNC–small Maf dimers. Much evidence exists in support of a role for CNC factors in EpRE-mediated transcription. Transgenic mice null for *nrf2* (*nrf2*^{−/−}) exhibited deficiencies in basal as well as butylated hydroxyanisole (BHA)-inducible expression of NAD(P)H quinone oxidoreductase 1 (NQO1) and glutathione S-transferase, as compared with their heterozygous littermates [14]. Impaired induction of oxidant-responsive genes and elevated sensitivity to oxidant toxicity has also been reported *in vitro* in peritoneal macrophages derived from *nrf2*^{−/−} mice [15]. Recently, Chan and Kwong [19] reported reduced expression of both the catalytic and modifier subunits of glutamate-cysteine ligase (GCLC and GCLM), the enzyme which catalyses the rate-limiting reaction in GSH synthesis, in fibroblasts derived from *nrf2*-null mice. Several investigators have observed activation of EpRE-, NF-E2- and MARE-con-

Abbreviations used: PDTC, pyrrolidinedithiocarbamate; PEITC, phenylethyl isothiocyanate; tBHQ, t-butylhydroquinone; EpRE, electrophile response element; CNC, Cap 'N Collar; MARE, Maf-recognition element; GCLM, glutamate-cysteine ligase modifier subunit; GCLC, glutamate-cysteine ligase catalytic subunit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RPA, RNase-protection analysis; AP, activator protein; ARE, antioxidant response element.

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taining promoter/reporter constructs by Nrf1 [10,20,21], Nrf2 [5,5a,10,20,22,23] and p45 [5,5a,7,20,24]. Because CNC proteins do not homodimerize and fail to bind DNA in the absence of a dimerization partner, it has been assumed that the involvement of CNC factors in EpRE-mediated transcription obviates the involvement of a dimerization partner as well. Some of the above studies [7,10,20,23,24] reported enhanced transcriptional activation when low amounts of small Maf proteins were co-transfected with the activating CNC factor. In all, however, increasing levels of small Maf inhibited both basal and CNC-protein-inducible promoter activity, a result attributed by several to a putative shift towards Maf–Maf homodimer formation. Although the ability of small Maf proteins to dimerize with CNC factors and bind to DNA-recognition elements has been demonstrated by numerous researchers in various experimental systems *in vitro*, the identification of small Maf proteins as the dimerization partners *in situ* remains somewhat controversial. This is due, in part, to the fact that several other transcription factors are also able to dimerize or interact with CNC factors [25–28]. Also worth noting with regard to small Maf involvement in stress response is the recent report by Hale et al. [29], which demonstrates an active MARE in the promoter of the p53 tumour-suppressor gene, the gene product of which is involved in the cellular response to DNA-damaging agents.

The response of the three small *maf* genes to oxidative stress and other insults is not well elucidated. Crawford et al. [30] reported increased expression of a *mafG*-like message (*adapt66*) in HA-1 hamster fibroblasts following exposure to H₂O₂. Consistent with this early report, Wild et al. [23] demonstrated increased *mafG* expression in HepG2 cells exposed to pyrrolidine-dithiocarbamate (PDTC) or β -naphthoflavone. More recently, Suzuki et al. [31] have proposed that small Mafs are involved in the cellular stress response, based on their observations that heavy metals induced *maf* expression in human HeLa cells and that heat shock induced *adapt66* expression in HA-1 hamster fibroblasts.

In order to better understand the stress-responsiveness of each of the three small *maf* genes, we have examined constitutive and PDTC-induced levels of endogenous *mafF*, *mafG* and *mafK* expression in several human cell lines derived from various tissues. Detailed characterization of the kinetics of induction of *mafF*, *mafG* and *mafK* expression in HepG2 cells exposed to PDTC, phenylethyl isothiocyanate (PEITC) and t-butylhydroquinone (tBHQ) is also reported.

MATERIALS AND METHODS

Reagents

PDTC, PEITC and tBHQ were purchased from Sigma. [α -³²P]dUTP was purchased from Amersham Pharmacia Biotech. Cell-culture materials were purchased from Gibco BRL unless otherwise noted. Acryl/bis, 19:1, solution was purchased from Amresco, Solon, OH, U.S.A.

Cell culture and treatments

The following cells were obtained from the A.T.C.C.: human lung carcinoma (A549; A.T.C.C. code CCL-185), adenovirus transformed human embryonic kidney (HEK-293; CRL-1573), human hepatocellular carcinoma (HepG2; HB-8056), human neuroblastoma (IMR-32; CCL-127), human leukaemia (K-562; CCL-243) and human rhabdomyosarcoma (RD; CCL-136). HEK-293, IMR-32 and RD cells were maintained in high-glucose Dulbecco's modified Eagle's medium, HepG2 cells in

low-glucose Dulbecco's modified Eagle's medium, K-562 cells in Iscove's modified Dulbecco's medium and A549 cells in α -minimal essential medium at 37 °C in a humidified atmosphere (3–4% CO₂). All media preparations were supplemented with 10% (v/v) fetal bovine serum (HyClone Laboratories, Logan, UT, U.S.A.), 2 mM L-glutamine and 50 μ g/ml gentamicin. Cells were harvested 0, 3, 6 or 12 h after treatment with fresh medium containing 100 μ M PDTC or vehicle control (water). Total RNA was extracted using TRI reagent (Molecular Research Center, Cincinnati, OH, U.S.A.) according to the manufacturer's instructions. This initial time course was used to determine when peak induction occurred in each cell line. The time of peak induction was then used to determine the duration of PDTC treatment in subsequent experiments.

For the extended time courses of induction in HepG2 cells, medium was replaced with fresh complete medium containing 100 μ M PDTC, 10 μ M PEITC, 50 μ M tBHQ or respective vehicle controls (water, ethanol or DMSO) once cells had reached 75–95% confluence. Cells were harvested 0, 1, 2, 3, 4, 6, 8, 12 and 24 h when treated with PDTC or PEITC, or 0, 4, 8, 10, 12, 14, 16, 18, 20, 24, 38 h when treated with tBHQ, after initial treatments.

RNase-protection analysis (RPA)

Portions of *mafF*, *mafG* and *mafK* were amplified from a genomic template using the following primers: *mafF*, 5'-GCCTCAGTCCCTCCCCAAAGTG-3' and 5'-ACCCCCAGGCCCAACAGAGG-3'; *mafG*, 5'-TTTGGCTGCCCTGTCCCTCTGTTT-3' and 5'-GCCTTCAAGCCCCGCCCTACTCTCA-3'; *mafK*, 5'-GGCGCAGGCAGCTCACACCAGGAA-3' and 5'-CCGGCAGCCCCACAGAGGAC-3'. The resulting PCR fragments were cloned into the PCR-Script SK(+) plasmid (Stratagene), and the absence of PCR-introduced mutations was confirmed by sequencing. The resulting plasmids were linearized with *PvuII* (*mafF*), *BglII* (*mafG*) or *NotI* (*mafK*) and used as templates for T7 polymerase in *in vitro*-transcription run-off reactions. An antisense probe against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was generated from pTRI-GAPDH (Ambion) and used as an internal control. 36B4 was also used as an internal control where indicated. Transcription reactions containing [α -³²P]dUTP were run for 3–5 h at 37 °C, with the exception of the reaction for 36B4, which was run at 15 °C. The three small *maf* probes were gel-purified (5% polyacrylamide/8 M urea), excised and eluted overnight at 37 °C into probe-elution buffer provided in the RPAIII kit (Ambion). The specific labelling of 36B4 and GAPDH was determined by trichloroacetic acid precipitation.

Expression levels of specific mRNAs were determined by RPA using the RPAIII kit (Ambion) according to manufacturer's procedure and as described previously [23]. Briefly, total RNA from treated cells was co-precipitated with an excess (determined empirically) of probe corresponding to the mRNA of interest as well as for an internal control (GAPDH or 36B4) and allowed to hybridize overnight at 60 °C. *maf* determinations were made using 100 μ g of total RNA/hybridization. Hybridized samples were digested with a 1:33 dilution of RNase A/T1 provided in the RPAIII kit. Protected fragments were resolved by denaturing PAGE (5% polyacrylamide/8 M urea). Gels were exposed on PhosphorImager cassettes and visualized. PhosphorImager scans were analysed by ImageQuant 1.2 (Molecular Dynamics).

Statistics

Data were corrected for loading, and fold inductions (treated/untreated) were determined at each time point. Fold inductions

were analysed by ANOVA and Fisher's protected least significant difference (PLSD). Reported *P* values are for fold inductions at the indicated time points as compared with fold inductions at 0 h.

RESULTS

Cell-type-specific differences in basal expression of small *mafs*

An RNase-protection assay designed to distinguish between the mRNAs corresponding to each of the small *mafs* was used to determine *mafF*, *mafG* and *mafK* expression in A549, HEK-293, HepG2, IMR-32, K-562 and RD cells. The constitutive levels of *mafF*, *mafG* and *mafK* expression varied among the cell lines examined (Figure 1). Constitutive *mafF* expression was highest in HepG2, K-562 and RD cells, intermediate in A549 and HEK-293 cells and low in IMR-32 cells. In contrast, *mafG* was observed to be highest in K-562, at intermediate levels in A549, HEK-293 and HepG2 cells and lowest in IMR-32 and RD cells. *mafK* was highest in A549 cells, high in HEK-293 and K-562 cells, at intermediate levels in HepG2 and RD cells and present at very low levels in IMR-32 cells.

Small *maf* expression in HepG2 cells exposed to PDTC, PEITC and tBHQ

The induction of *mafF*, *mafG* and *mafK* expression by PDTC, PEITC and tBHQ was examined in detail in HepG2 cells. All three small *maf* messages were increased in HepG2 cells exposed to 100 μ M PDTC. *mafF*, *mafG* and *mafK* were induced to a statistically significant extent as early as 2 h after PDTC treatment and returned to basal levels 8 h after treatment. Although the kinetics of induction did not appear to vary among the three small *mafs*, the magnitude of induction did (Figure 2); *mafF* was induced $\approx$ 5-fold, *mafK* $\approx$ 4-fold and *mafG* $\approx$ 2-fold. As was observed following PDTC treatment, all three small *maf* mRNAs were induced in HepG2 cells exposed to 10 μ M PEITC

Figure 1 Cell-type-specific variations in small *maf* constitutive expression

RNase-protection assays are shown for each of the small *mafs*. M denotes molecular-mass markers. Results are representative of three separate experiments. Lanes labelled 36B4 only, GAPDH only, *mafF* only, *mafG* only or *mafK* only contain RNA from HepG2 cells protected by the single indicated probe.

Figure 2 PDTC-induced expression of *mafF*, *mafG* and *mafK* in HepG2 cells

The time-dependent expression of *mafF*, *mafG* and *mafK* in HepG2 cells exposed to 100 μ M PDTC was determined by RPA as described in the Materials and methods section. *GAPDH* expression was used as an internal control. (A) RPA PhosphorImages are representative of at least three separate experiments. (B) Data are presented as means $\pm$ S.E.M. ($n = 3$). Increases in *mafF*, *mafG* and *mafK* messages were statistically significant ($P < 0.05$; compared with 0 h) 2, 3 and 4 h after treatment; *mafG* mRNA remained significantly elevated 6 h post-treatment.

(Figure 3). The relative magnitudes of induction (*mafF* > *mafK* > *mafG*) were also similar after PEITC treatment, although slight differences in the kinetics of induction were observed. *mafF* and *mafG* were elevated to a statistically significant extent by 3 h and had returned to basal levels 24 h after treatment. The induction of *mafK* was slightly delayed as compared with that of *mafF* or *mafG*, but had reached statistical significance 6 h after treatment with PEITC (Figure 2). None of the transcripts were markedly induced in HepG2 cells exposed to 50 μ M tBHQ at any time point (Figure 4).

Cell-type-specific differences in PDTC-inducible expression of small *mafs*

Because basal expression of the small *mafs* varied among cell lines, we further investigated whether the PDTC responsiveness of *mafF*, *mafG* and *mafK* would also differ among cell lines. To examine cell-line-specific differences in small *maf* induction, A549, HEK-293, HepG2, IMR-32, K-562 and RD cells were treated for 0, 3, 6 and 12 h with 100 μ M PDTC or a vehicle control. Preliminary analysis demonstrated that maximal induction for all the small *mafs* was seen at 3 h in HepG2 and RD

Figure 3 PEITC-induced expression of *mafF*, *mafG* and *mafK* in HepG2 cells

The time-dependent expression of *mafF*, *mafG* and *mafK* in HepG2 cells exposed to 10 μ M PEITC was determined by RPA as described in the Materials and methods section. *GAPDH* or *36B4* expression was used as an internal control. **(A)** Phosphorimages are representative of at least three separate experiments. **(B)** Data are presented as means $\pm$ S.E.M. ($n = 3$). Increases in all three small *maf* messages were statistically significant ($P < 0.05$; compared with 0 h) 6 and 8 h after treatment, and *mafG* and *mafK* mRNAs remained significantly elevated 12 h after treatment. Both *mafF* and *mafG* were significantly induced as early as 3 h after treatment.

cells and at 6 h in A549, HEK-293, IMR-32 and K-562 cells (results not shown). *mafF* was induced to the highest levels in all cell lines examined (Figure 5), although there were dramatic cell-type-specific differences in the magnitude of induction, which ranged from 25-fold in RD cells to 5-fold in IMR-32 and K-562 cells. *mafG* and *mafK* were induced to comparable levels in HEK-293, K-562 and RD cells (2–3-fold in all three cell lines). In A549 cells, *mafK* was induced 3-fold whereas *mafG* was not induced. In IMR 32 cells, *mafG* was induced 3.5-fold, and *mafK* was not induced.

PDTC-mediated increases in *mafF* expression were transcriptionally mediated

Induction of *mafF* expression following PDTC exposure was greater than *mafG* and *mafK* in all cell lines examined, except IMR-32. In order to determine whether this induction was transcriptionally mediated, HepG2 cells were pre-treated with

Figure 4 *mafF*, *mafG* and *mafK* expression in HepG2 cells exposed to tBHQ

The time-dependent expression of *mafF*, *mafG* and *mafK* in HepG2 cells exposed to 50 μ M tBHQ was determined by RPA as described in the Materials and methods section. *GAPDH* or *36B4* expression was used as an internal control. Data are presented as means $\pm$ S.E.M. ($n = 3$) or the mean of two separate determinations.

10 μ g/ml actinomycin D for 1–1.5 h prior to PDTC exposure. Actinomycin D not only prevented PDTC-induced increases in *mafF* message, but also resulted in a slight decrease in basal levels (Figure 6).

DISCUSSION

Maf proteins are highly conserved across species. There is also remarkable amino acid sequence similarity among the three small Maf proteins (reviewed in [1]), which has led to speculation regarding functional redundancy. Indeed, in various *in vitro* assays MafF, MafG and MafK are functionally interchangeable [4,7], and overexpression of one small Maf can, in some cases, compensate for the loss of another in transgenic animals [13,32]. *mafF*-null [33], *mafK*-null [32] and *mafF::mafK*-null [34] mice did not demonstrate any phenotype discernable from the wild type. By contrast, *mafG*-null mice were smaller than their heterozygous littermates and demonstrated clear haematological and neurological abnormalities. Elimination of *mafF* expression against a *mafG*-null background resulted in only a slight exacerbation of the *mafG*-null phenotype, whereas in *mafK::mafG*-null mice the exacerbation of the *mafG*-null phenotype was severe [34]. The above studies are consistent with partial redundancy among MafF, MafG and MafK, but also indicate a lack of complete functional redundancy among small Mafs *in vivo*. Additionally, the three small Mafs exhibit distinct spatial and temporal expression profiles in developing mouse embryos [32,33], as well as differing patterns of tissue-specific expression in adult mice [33,34]. These differences in small Maf expression profiles hint at varied mechanisms of regulation among the three genes. We observed differences in the degree to which each small Maf was expressed and induced within a given cell line. This observation is consistent with reported expression profiles in adult mice and provides further support for alternative regulatory mechanisms of gene expression and response among the three individual small *maf* genes. The cell-line-specific differences in expression of *mafF*, *mafG* and *mafK* may reflect the tissue type of origin, degree of cell differentiation, varied proliferation or a combination of these and other factors.

Figure 5 Cell-type-specific variations in induction of small *maf* expression by PDTC

(A) Representative RNase-protection assays showing induction of small *mafs* after treatment with 100 μ M PDTC for 3 h (RD) or 6 h (A549, HEK-293, IMR-32 and K-562). (B) Fold inductions over untreated controls. Results are the means $\pm$ S.E.M. from three separate experiments.

PDTC, PEITC and tBHQ are compounds that have been associated with the induction of stress-response genes, probably via activation of EpRE/ARE [17,35–38]. Since small Mafs are thought to play a role in EpRE-mediated transcription, we examined the ability of these EpRE activators to alter small *maf* expression. Although both PDTC and PEITC induced *mafF*, *mafG* and *mafK* expression in HepG2 cells, tBHQ did not. Induction was fairly rapid and transient. Differences in the ability of tBHQ as compared with PDTC and PEITC to induce small *maf* expression may reflect differences in the nature of the redox or stress signal generated by these compounds. PDTC

Figure 6 Actinomycin D inhibited PDTC-mediated induction of *mafF*

HepG2 cells were treated with 10 μ g/ml actinomycin D (ActD) for 1–1.5 h prior to PDTC treatment. The expression of *mafF* 4–4.5 h after PDTC treatment was determined by RPA as described in the Materials and methods section. (A) The PhosphorImage is representative of three separate experiments. (B) Bands were quantified and indicate the ratio of *mafF*/36B4 for each lane. Data are presented as means $\pm$ S.E.M. from three separate experiments.

exhibits both antioxidant and pro-oxidant characteristics, depending on the experimental system. PDTC's cytotoxicity in thymocytes has been attributed to its ability to chelate redox-active copper and shuttle it into the cell, where it can participate in the generation of reactive oxygen species [39]. Alternatively, PDTC itself can be oxidized to ultimately form the corresponding thiuram disulphide [40]. Thiuram disulphides are reactive compounds capable of oxidizing GSH [40] and other reduced thiols [41]. Increased expression of *GCLC* and *GCLM* in HepG2 cells exposed to PDTC was associated with elevated levels of GSSG, but did not appear to be mediated by increased intracellular reactive oxygen species [42]. The potency of various isothiocyanates to induce EpRE-mediated transcription has been correlated with their ability to form GSH conjugates, which accumulate intracellularly and can be further metabolized *in vivo* to generate thiocarbamates [43–45]. The fact that PEITC elicited a pattern of small *maf* induction similar to that observed following PDTC exposure may be partially attributable to its metabolism to a thiocarbamate. In contrast with what Wild and Mulcahy [42] observed in HepG2 cells treated with PDTC, Yamane et al. [46] reported increased intracellular reactive oxygen species in rat hepatoma cells treated with tBHQ, an observation also reported by Pinkus et al. [47] in HepG2 cells. Pinkus et al. [47] further

reported induced transcription from the mouse GSTY α promoter as well as binding *in vitro* to an AP-1 site following PDTC or tBHQ exposure, whereas only tBHQ and not PDTC induced binding to a nuclear factor κ B consensus sequence. This difference supports the possibility that, while both PDTC and tBHQ are oxidants and effective inducers of EpRE activity, these two compounds may generate different signals with regard to oxidative stress response, and hence exert different effects on *maf* gene expression.

Of the three small *mafs*, *mafF* appeared to be the most responsive following PDTC exposure in all cell lines examined and in HepG2 cells exposed to either PDTC or PEITC. It is interesting to note that previous reports in the literature have focused on the inducibility of *mafG* [23,30,31], although Suzuki et al. [31] did report increases in *maf*-related messages that were not specifically identified. The ability of actinomycin D to eliminate PDTC-induced increases in *mafF* mRNA levels suggests that this increase was transcriptionally mediated. The actinomycin D-mediated reduction in basal levels is consistent with a labile steady state accomplished by rapid induction and rapid degradation of *mafF*, which may also be reflected in the sharp induction and rapid return to basal levels observed in HepG2 cells exposed to PDTC or PEITC.

We conclude that small *maf* expression varies among human cell lines, as does the magnitude of small *maf* response to PDTC treatment. All three small *mafs* appear to be responsive to PDTC and PEITC, but tBHQ failed to elicit a marked effect in expression of any of the three small *mafs*. Because tBHQ, PDTC and PEITC are all effective inducers of EpRE activity, we conclude that up-regulation of small *mafs* is not an absolute requirement for EpRE-mediated gene expression. Of the three small *mafs*, *mafF* was induced to the greatest extent in all cell lines examined and, in HepG2 cells at least, this induction was transcriptionally mediated. These results suggest that, even within a single cell line, expression of *mafF*, *mafG* and *mafK* genes may be regulated by distinct mechanisms.

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