Hormeza – primer odgovora astrocitov po izpostavljenosti etanolu

Hormesis – an example of astrocyte response after ethanol exposure

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Izvleček

Namen: *Namen dela je bil opisati hormezo in predstaviti pregled hormetičnih odgovorov astroglialnih celic po izpostavljenosti različnim stresorjem. Predstavili smo primer odgovora astrocitov na akutno in kronično izpostavljenost etanolu, ki ima značilnosti hormeze.*

Metode: *Kot eksperimentalni model smo uporabili celične culture astrocitov možganske skorje novorojenih podgan. Celice smo izpostavili etanolu in njegovemu primarnemu metabolitu acetaldehidu za 24 ur in sedem dni. Po obravnavi celic smo v vzorcih določili vsebnost proteinov in s pomočjo encimsko–imunskega testa izmerili količino sproščenega interlevina–6 (IL–6).*

Rezultati: *Izpostavljenost astroglialnih celic etanolu ali acetaldehidu v nizkih koncentracijah vodi do povečanja*

Abstract

Purpose: *In the present work we describe hormesis and review documented hormetic responses in astroglial cells after exposure to different stressors. We present an example of the astrocyte response to acute and chronic ethanol exposure with hormetic characteristics.*

Methods: *As an experimental model, newborn rat cortical astrocytes in culture were used. The cells were exposed to ethanol or the primary metabolite of ethanol (acetaldehyde) for 24 h or 7 days. After treatment, the protein content was determined, and the IL–6 levels in the culture medium were measured using an enzyme–linked immunoassay.*

Results: *Treatment of astroglial cells with ethanol or acetaldehyde led to enhanced protein content* *vsebnosti proteinov in stimulira nastajanje IL–6, pri visokih koncentacijah pa se pojavi zmanjšanje vsebnosti proteinov in inhibicija nastajanja IL–6. Kronična izpostavljenost je za astrocite pri obeh snoveh bolj toksična kot akutna. Acetaldehid je bolj toksičen kot etanol.*

Zaključek: *Etanol in acetaldehid predstavljata za astrocite v kulturi stres in vzpodbudita tipični hormetični odgovor, tako pri akutni kot tudi pri krončni izpostavljenosti.*

and stimulated IL–6 production in the low concentration zone followed by diminished protein content and an inhibition of IL–6 production at higher concentrations. Chronic exposure of astrocytes was more toxic than acute exposure for both compounds, and acetaldehyde was more toxic compared to ethanol.

Conclusion: *Ethanol and acetaldehyde represent stressors for cultured astrocytes and evoke a typical hormetic response after acute and chronic exposure.*

INTRODUCTION

Astrocytes are essential for maintaining a healthy and well–functioning brain. Astrocytes face the synapses, send end–foot processes that enwrap the brain capillaries, and form an extensive network that is interconnected by gap junctions. Astrocytes have the potential to impact essentially all aspects of neuronal function through regulation of blood flow, provision of energy substrates, or by influencing synaptic function and plasticity. Moreover, astrocytes also protect and aid the brain in the functional recovery from injuries. The activation of glial cells in the CNS is the first defense mechanism against pathologic abnormalities that occur in neurodegenerative diseases (1). A body of data indicates that astrocytes display adaptive responses when exposed to a diverse range of stressor agents, including a broad range of toxic metals, the antibiotic ciprofloxacin, and the antitumor agent ET–18–OCH (ET), a synthetic analogue of 2–lysophophatidylcholine (for review, see Ref. 2). In all of these cases, the adaptive responses are expressed within the context of a biphasic dose response, which is referred to as an example of hormesis (3–5). Many biological disciplines assess specific aspects of this non–linearity phenomenon (hormesis), and several terms attribute these biological responses to the plethora of possible stressors with respect to diverse endpoints in varied biological models. For example, some terms address the shape of the dose–response curve, such as a β–curve, biphasic, bell–shaped, U–shaped, inverted–U shaped, J-shaped, diphasic, bitonic, bimodal, bidirectional, sinusoidal, subsidy gradient, functional antagonism, dual response, non–monotonic, and stimulatory inhibitory (6). Terms, such as autoprotection, heteroprotection, adaptive response, pre–conditioning, hormesis, xenohormesis, and paradoxic have characterized the shape of the dose–response patterns mentioned above when

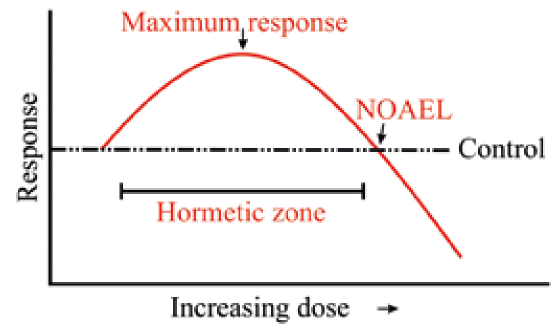

Figure 1. *An example of a dose–response relation depicting the quantitative features of hormesis. The figure illustrates the average maximum stimulation range, no observed adverse effect level (NOAEL), and the typical width of the stimulation range.*

low doses elicit an adaptive response by the cell or organism (7, 8).

The hormetic dose–response is described as stimulation in the low–dose zone, followed by an inhibitory response at higher doses. The magnitude of the stimulatory response at maximum is typically modest, being only 30%–60% greater than that of the control response (Figure 1). The strong majority of stimulatory responses are less than twice the control value. This is the most distinguishing characteristic of the hormetic dose–response, being the most consistent and reliable feature. The width of the stimulatory response is typically within 100–fold of the zero equivalent point, the dose at which the response changes from stimulation to inhibition (i.e., the threshold value). In a small proportion of the hormetic dose responses analyzed to date, a very broad stimulatory dose–response range has been noted, exceeding 1000–fold. The implications of having a wide stimulatory zone may be clinically significant because in the case of drugs, the stimulatory zone may define the therapeutic window (6, 7).

In the present work we demonstrate a typical hormetic response in protein content and interleukin–6 (IL–6) secretion obtained in cultured astrocytes after acute and long–term exposure to ethanol and the primary metabolite of ethanol (acetaldehyde).

MATERIALS AND METHODS

Materials

L–15 Leibowitz medium, foetal bovine serum (FBS), Dulbecco's modified Eagle medium, and Ham's nutrient mixture F–12 (DMEM / F12), penicilin–streptomicyn (10,000 IU/ml – 10,000 UG/ml; P/S), and Dulbeco's phosphate buffered saline (PBS) were purchased from Gibco BRL (Life Technologies, Paisley, Scotland). Ethanol and acetaldehyde were obtained from Merck (Darmstadt, Germany). Bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The Bio–Rad protein assay was purchased from Bio–Rad Laboratories (Munich, Germany). An Endogen Rat IL–6 ELISA Kit was obtained from Pierce Biotechnology (Rockford, IL, USA).

Animals

Newborn Wistar rats (postnatal day 2) were obtained from our own breeding colony. The animals were maintained under constant environmental conditions, with an ambient temperature of 22±1° C, a relative humidity of 55±10 %, and a natural light–dark cycle. The breeding colony was kept in Ehret type–4 cages (Emmendingen, Germany). The bedding material used was Lignocel 3/4. The colony received a standard rodent diet (Altromin, Lage, Germany), and had free access to food and water. We used four newborn animals in each experiment.

All the animal procedures were approved by the National Animal Ethical Committee of the Republic of Slovenia (license number, 323–02–232/2005/2) and were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123).

Astrocyte culture preparation

Primary cultures of rat cortical astrocytes were prepared from the brains of newborn Wistar rats. The newborn rats (postnatal day 2) were decapitated and the brains were removed aseptically. After removal of the meninges, the cortices were transferred to a Petri dish containing L–15 (Leibowitz) medium. The cortices were then mechanically dissociated into 10 ml of culture medium, consisting of DMEM/F12 (1:1), 10% (vol/vol) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell suspension was triturated and plated into tissue–culture flasks. The cells were grown at 37° C in a water–saturated air environment, containing 10 % CO_2. The cultures were purified by shaking at 150 rpm for 18 h when confluent to remove microglial cells. After shaking, the medium was changed and the cells were trypsinized and cultured for 24 h in the presence of 10 μM cytosine arabinoside that only allowed growth of astrocytes. After reaching confluence again, the cells were sub–cultured onto 35–mm Petri dishes for treatment with ethanol or acetaldehyde.

Treatment of the cells

a) Acute treatment: After the cultures became confluent, the culture medium was replaced with 1 ml of fresh medium and the cells were treated with different concentrations of ethanol or acetaldehyde for 24 h.

b) Long–term treatment: After the plating onto Petri dishes, the cells were grown in media containing different concentrations of ethanol or acetaldehyde for the next 7 days. To minimize the decline of the ethanol and acetaldehyde concentrations in the culture medium due to evaporation, the media were changed every 48 h and tightly closed in the Petri dishes, which allowed a reduction in the ethanol and acetaldehyde concentrations in the culture medium of < 10 %.

The concentrations of ethanol and acetaldehyde used in the present study were based on our previous studies in which a dose–response relationship for ethanol and acetaldehyde on cell viability and cell proliferation was studied (9). Only concentrations below the threshold of a significant decrease in viability were used.

The experiments were performed under lipopolysaccharide–free conditions. The control cells were grown under the same conditions in the absence of ethanol or acetaldehyde.

Protein determination

After the treatment, the culture medium was removed and the cells from individual dishes were harvested and used for protein determination using Bio–Rad protein assay protocol.

IL–6 secretion determination

After the treatment, the culture medium was collected, frozen, and used for IL–6 determinations. The IL–6 levels in the culture media were determined by enzyme immunoassay using the Pierce Biotechnology IL–6 ELISA protocol.

Statistical Analysis

The results are shown as the mean ± the standard error of the mean (SEM) of three independent assays. One–way ANOVA with a Tukey post–test were used to calculate the significance of the differences between the means. A p–value < 0.05 was considered to be statistically significant.

RESULTS

The influence of ethanol and acetaldehyde on protein content

Acute exposure of cultured astrocytes to ethanol for

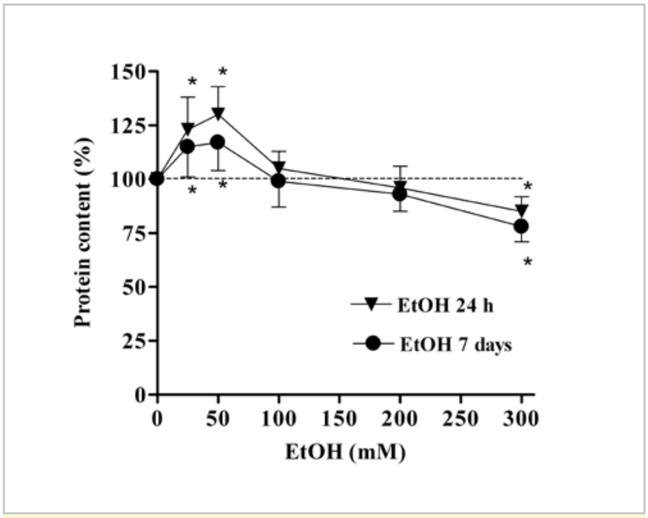

*Figure 2. The influence of different concentrations of ethanol (EtOH) on the protein content in the cell culture, after 24 h and 7 days of exposure. Each bar is the mean ± SEM of three independent determinations. *p < 0.05 versus control cells.*

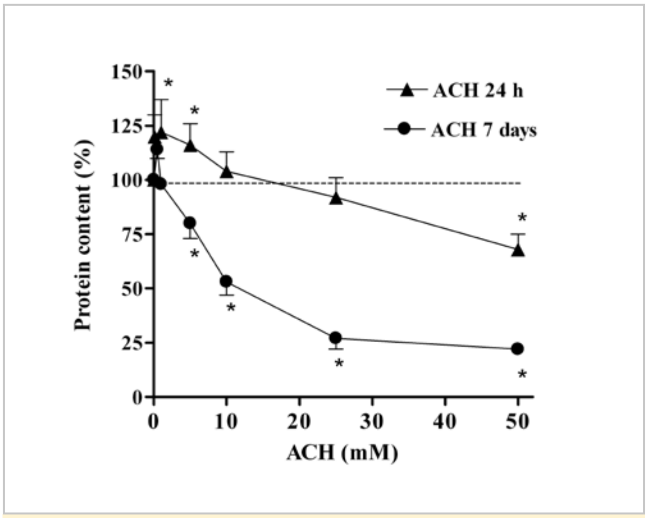

*Figure 3. The influence of different concentrations of acetaldehyde (ACH) on the protein content in the cell culture, after 24 h and 7 days of exposure. Each bar is the mean ± SEM of three independent determinations. *p < 0.05 versus control cells.*

24 h did not influence the number of the cells in the culture (data not shown). In contrast, the protein content in the cultures showed a biphasic course when evoked by ethanol (Figure 2). Low concentrations of ethanol significantly stimulated protein production, with the maximum protein content at 50 mM ethanol after acute and long–term exposure. Acute exposure to ethanol evoked a higher peak of protein content in comparison to long–term exposure. Concentrations of ethanol > 50 mM diminished protein content, with no observed adverse effect level (NOAEL) at 100 mM ethanol. Concentrations > 100 mM significantly diminished protein content in comparison to control cells. Similarly, acetaldehyde enhanced protein content at low concentrations with a maximum at 0.5 mM concentration. NOAEL was observed at 1mM acetaldehyde. At concentrations > 1mM, acetaldehyde diminished the protein content in comparison to control cells (Figure 3). Acute exposure of the cultured astrocytes to ethanol or acetaldehyde induced a stronger hormetic response than long–term exposure.

The influence of ethanol and acetaldehyde on IL–6 secretion

In the next set of experiments, we determined the influence of different concentrations of ethanol and acetaldehyde on IL–6 secretion from cultured astrocytes after acute and long–term exposure. We found that low concentrations of ethanol strongly stimulated IL–6 secretion. After 24 h of exposure, the maximal increase in IL–6 secretion was observed

Table 1: *Hormetic response in astroglial cells induced by various substances*

Substance	Experimental model	Effect	Reference
Chloroquine	Primary astrocyte culture	GFAP* content	(10)
6–hydroxydopamine	Primary astrocyte culture	GFAP content	(10)
Toluene	Primary astrocyte culture	GFAP content	(10)
Aluminium chloride	Primary astrocyte culture	GFAP content	(10)
Mercury chloride	Primary astrocyte culture C6 glioma cells U373MG cell line	GFAP content MTT conversion Mitochondrial deydrogenase activity	(10) (11) (12)
2,4–dinitrophenol	Primary astrocyte culture	MTT** conversion	(11)
Ethanol	Primary astrocyte culture	MTT conversion	(11)
Triethilin	Primary astrocyte culture	MTT conversion	(11)
Aluminium chloride	C6 glioma cells	MTT conversion	(11)
Lead	C6 glioma cells	MTT conversion	(11)
Cadmium chloride	C6 glioma cells	MTT conversion	(11)
Chloroquine	C6 glioma cells	MTT conversion	(11)
Methyl mercury	U373MG cell line	Mitochondrial deydrogenase activity	(12)
IL–4	C6 glioma cells	DNA synthesis	(13)
Ciprofloxacin	Primary astrocyte culture	Lysosomal membrane damage	(5)
ET–18–OCH (ET) : synthetic analogue of 2–lysophophatidylcholine	Primary astrocyte culture	Glutamine synthetase activity	(14)
Endozepin	Primary astrocyte culture	Thymidine incorporation	(15)
Riluzole	Primary astrocyte culture	Glutamate uptake	(16)

Legend:

**GFAP – glial fibrillary acid protein, an indicator of a toxic response in astrocytes*

***MTT – methallothionien, a biomarker of neurotoxicity*

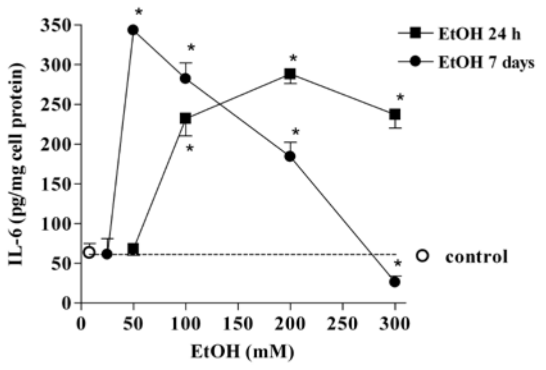

Figure 4. *The influence of different ethanol (EtOH) concentrations after 24 h and 7 days of exposure on IL–6 secretion from cultured astrocytes. Each point is the mean ± SEM of three independent determinations.* $^{*}p < 0.05$ *versus control cells.*

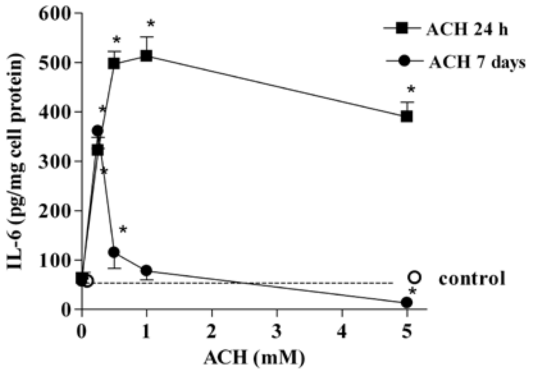

Figure 5. *The influence of different acetaldehyde (ACH) concentrations on IL–6 secretion from cultured astrocytes after 24 h or 7 days of exposure. Each point is the mean ± SEM of three independent determinations.* $^{*}p < 0.05$ *versus control cells.*

at 200 mM ethanol. At higher concentrations, the secretion of IL–6 began to decrease. With long–term exposure, the maximal secretion of IL–6 was observed at 50 mM ethanol. At concentrations > 50 mM, the secretion of IL–6 began to decrease, with NOAEL at 300 mM (Figure 4).

Similarly, after acute exposure, acetaldehyde stimulated IL–6 secretion in cultured astrocytes with the maximum reached at 1 mM acetaldehyde. Higher concentrations of acetaldehyde diminished IL–6 secretion. With long–term exposure, acetaldehyde caused stimulation of IL–secretion much earlier, and at 1 mM acethaldehyde NOAEL was reached (Figure 5).

DISCUSSION

Astrocytes are able to display different adaptive responses when exposed to stressors and/or toxic agents, including a broad range of toxic metals and other xenobiotics, such as the antibiotic ciprofloxacin and the anti–tumor agent ET (2). In all cases, the adaptive responses are expressed within the context of a biphasic dose response as an example of hormesis (3–5). It is important to note that initial stimulation in the low–dose zone, followed by an inhibitory response at higher doses, is considered to be an adverse effect (3). However, in the late 1990s investigators re–interpreted earlier findings and concluded that the low–dose stimulation component of the biphasic dose responses was adaptive in nature, and incorporated the findings into a hormetic dose–response framework (2; Table 1).

Of further interest is that several endogenious substances are also able to evoke a hormetic response in glial cells. In this manner, endozepine peptides may enhance glial cell proliferation, not only during developmental processes, but also during brain tumor proliferation. Numerous other examples exist in which endogenous agents enhance tumor cell proliferation, including brain tumor cell lines, which is consistent with the hormetic dose response (17, 18).

The findings that hormetic dose responses occur and are widespread in astroglial cells are consistent with findings with neurons in primary cell cultures and neuronal cell lines (2, 7).

Ethanol has an extensive array of actions on astrocytes, transforming astrocytes into activated, potentially injurious cells with negative consequences to neuronal function and survival, and to brain function (19). In the present work we showed that ethanol and the primary metabolite of ethanol (acetaldehyde) are able to evoke a biphasic dose response in cultured astrocytes. The treatment of astroglial cells with ethanol or acetaldehyde led to enhanced protein content and stimulated IL–6 production in the low concentration zone, followed by diminished protein content and inhibition of IL–6 production at higher concentrations of both compounds. Long–term exposure of astrocytes is more toxic than acute exposure for both compounds, and acetaldehyde was shown to be more toxic compared to ethanol. Our results are in agreement with the findings of Pentreath and Salmon (3), who reported that most gliotoxic agents have a biphasic dose–response curve; specifically, the response increases at low, sub–toxic doses, followed by a decrease at higher, cytotoxic doses. The concentration range over which a biphasic response occurs could vary by several orders of magnitude for different markers of the same toxicant.

Ethanol has several targets in astrocytes and other cell types, impairing cellular redox status, cell growth and differentiation, interfering with the stimulatory effect of trophic factors, or altering the expression of cytoskeletal proteins (19). In addition, ethanol induces astroglial activation, associated with up–regulation of several pro–inflammatory cytokines that contribute to neuroinflammation, neurodegeneration, and cell apoptosis (20). Inflammation is primarily a protective response of the target organism to a noxis. In contrast, excessive or long–lasting inflammation is often followed by degenerative processes. The stimulatory effect of ethanol and acetaldehyde on IL–6 secretion appears to be involved in neuroregenerative and survival processes, as well as in neurodegeneration. The resultinghormestic dose–response relationship indicates that higher concentrations and long–term exposure could lead to neurodegeneration, whereas low concentrations may be neuroprotective.

The possibility that neurotoxic agents could induce adaptive/protective responses in neurons and astroglial cells was not believed possible by many neurotoxicologists less than a decade ago. This newly appreciated adaptive response concept is now being actively assessed to develop practical ways to slow and possibly reverse the progress of neurodegerative diseases (8, 21). While there has been a dominant perspective that neurotoxins may be harmful at any dose, this is actually not the case in the assessment of astrocyte responses. The analysis of reports, as well as our data, demonstrates that neurotoxin–induced adaptive responses are commonly observed across a broad spectrum of highly toxic agents, including lead, methyl mercury, ethanol, numerous other xenobiotics, and endogenous substances. Highly toxic agents could therefore induce protective responses at low doses via hormetic mechanisms that should not be surprising, even if it is likely to be controversial.

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