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Research Report

The concept of "aldehyde load" in neurodegenerative mechanisms: Cytotoxicity of the polyamine degradation products hydrogen peroxide, acrolein, 3-aminopropanal, 3-acetamidopropanal and 4-aminobutanal in a retinal ganglion cell line

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ABSTRACT

In neurodegenerative diseases augmented polyamine metabolism results in the generation of hydrogen peroxide and a number of reactive aldehydes that participate in the death of compromised tissue. The major aldehydes produced by polyamine oxidase and amine oxidases include the 2-alkenal acrolein, the acetoamidoaldehyde 3-acetamidopropanal (3-AAP) and the aminoaldehydes 3-aminopropanal (3-AP) and 4-aminobutanal (4-AB). Using retinal ganglion cell (E1A-NR.3) cultures, we confirmed the cytotoxicity of acrolein and 3-AP. For the first time we also demonstrated the cytotoxicity of 4-AB and the lack of toxicity of 3-AAP. Our data with 3-AAP, a product of N-acetylspermine and N-acetylspermidine metabolism, indicate that the aldehyde function of aminoaldehydes is insufficient to express toxicity since the free amino group of aminoaldehydes is also required to gain access to lysosomes where their cytotoxic actions are expressed via leakage of cathepsins that compromise mitochondrial integrity. Metabolism of 3-AP to β-alanine by aldehyde dehydrogenase was also evaluated in retinal ganglion cell cultures and found to proceed at a linear rate of 24.3±1 nmol/mg protein/h. These are the first data demonstrating the dynamic cellular detoxification of 3-AP by neural cells and support the concept that decrements in aldehyde elimination leading to an increase in "aldehyde load" may play pivotal roles in the development and progression of neurodegenerative diseases such as Alzheimer's disease, multiple sclerosis and Parkinson's disease.

1. Introduction

Increased brain and CSF levels of reactive aldehydes, free and/or protein bound, have been reported for various neurodegenerative diseases (Zarkovic, 2003). These have been used as indices of "oxidative stress". However, there are multiple metabolic sources of reactive aldehydes, including lipid peroxidation, carbohydrate glycation reactions, myeloperoxidase metabolism of the amino acids tyrosine, serine and threonine, amine oxidase metabolism of

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monoamines, lysyl oxidase actions in the ECM, intermediary metabolism of carbohydrates, amino acids and phospholipids and polyamine metabolism. While all of these sources may play roles in neurodegenerative diseases, polyamine metabolism is clearly a large metabolic source of reactive aldehydes that is augmented in neurological disorders and preclinical models of neurodegeneration. The prevalent reactive aldehyde metabolite of polyamine metabolism (Cervelli et al., 2004; Murray-Stewart et al., 2002; Seiler, 1995, 2000, 2004) appears to be 3-aminopropanal (3-AP, Table 1) which is a product of spermine, spermidine and 1,3-propanediamine oxidation by FAD-dependent amine oxidases [EC 1.4.3.4; monoamine oxidase (MAO), spermine oxidase and polyamine oxidase (PAO)] and copper-dependent amine oxidases [EC 1.4.3.6; diamine oxidase (DAO) and semicarbazide-sensitive amine oxidase (SSAO)]. Dramatic increases in the levels of 3-AP have been shown to precede ischemia-induced delayed cell death (Ivanova et al., 1998) and delayed neuronal cell death, not involving ischemia, in the rat trimethyltin model of hippocampal CA3 neurodegeneration (Wood et al., 2006b). Mechanistic studies have characterized the cytotoxic actions of 3-AP in the human neuroblastoma cell line HTB11 (Ivanova et al., 1998; Yu et al., 2004); in the human glioma cell lines HTB14 (Ivanova et al., 1998) and D384 (Li et al., 2003); in the rat retinal ganglion cell line E1A-NR.3 (Wood et al., 2006b); and in the murine macrophage cell line J774 (Yu et al., 2003). These studies have shown that the aldehyde function is required for cytotoxicity and that the amino function is required for uptake into lysosomes where cytotoxicity is initiated (Li et al., 2003; Yu et al., 2003, 2004). With high concentrations of 3-AP, frank lysosomal rupture can occur leading to cellular necrosis (Li et al., 2003). In contrast, lesser insults activate the intrinsic apoptotic pathway via graded lysosomal rupture and leakage of cathepsins which in turn compromise mitochondrial integrity leading to cytochrome *c* release into the cytoplasm and activation of the caspase cascade (Li et al., 2003; Yu et al., 2003, 2004).

In addition to the generation of reactive aldehydes, alterations in their metabolism can contribute to aldehyde toxicity in neurodegenerative diseases. Rapid and large decrements in the metabolic capacity to eliminate aldehydes, as in ischemia–reperfusion injury (Ivanova et al., 1998), can lead to dramatic and sustained increases in the levels of aminoaldehydes. In Alzheimer's disease which is characterized by aldehyde accumulation starting at early phases of the disease, including mild cognitive impairment (Ahmed et al., 2005, Williams et al., 2006), a more chronic and gradual disease course over several decades may involve decreases in the metabolic capabilities to detoxify aldehydes that possess the potential to contribute to neurodegeneration. The major metabolic pathways involved in the inactivation of reactive aldehydes include mercapturic acid formation via glutathione-S-transferases (GST, EC 2.5.1.18, Linhart et al., 1996; He et al., 1998; Martinez-Lara et al., 2003; Sidell et al., 2003), oxidation to carboxylic acids via aldehyde dehydrogenases (ALDH, EC 1.2.1.-, Kikonyogo and Pietruszko, 1996; Murphy et al., 2003; Meyer et al., 2004; Rooke et al., 2000) and reduction to alcohols by aldo-keto reductases (AKR, EC 1.1.1.-, Picklo et al., 2001; Matsunaga et al., 2006). Previous studies of 4-hydroxynonenal metabolism by vascular smooth muscle cells have demonstrated that only a small fraction of the free aldehyde pool escapes cellular metabolism (Srivastava et al., 2001). These investigators suggested that as a result of the very rapid metabolism of aldehydes, measurements of "aldehyde load", via measurements of free and protein-bound aldehyde, dramatically underestimate the extent of reactive aldehyde generation *in vitro* and *in vivo*. We therefore undertook preliminary studies of the metabolic dynamics of an excessive "aldehyde load" in a rat retinal ganglion cell line to evaluate the dynamics of 3-AP detoxification. Previous studies of acrolein and 4-hydroxynonenal have demonstrated that these aldehydes are mainly metabolized by GST and ALDH (Srivastava et al., 2001; Meyer et al., 2004). Metabolism of 3-AP has not been studied and since this aldehyde does not have an electrophilic center, it is not a substrate for GST. We therefore examined the metabolism of 3-AP by ALDH.

While 3-AP is the major metabolic product of polyamine metabolism, other aldehyde products (Seiler, 2004) include the aminoaldehyde 4-aminobutanal (4-AB, Table 1), the acetoamidoaldehyde 3-acetamidopropanal (3-AAP, Table 1), which is produced from oxidation of acetylated polyamines, and the reactive 2-alkenal, acrolein (Table 1). The cytotoxicity of 3-AP and acrolein has been well characterized, however, that of 4-AB and 3-AAP has not. We therefore synthesized both aldehyde products of polyamine metabolism and compared their cytotoxicity to that of 3-AP and acrolein in a rat retinal ganglion cell line. We used hydrogen peroxide (H_2O_2) as a further comparator since it is also a product of polyamine oxidation.

Table 1 – Polyamine sources of H_2O_2 and the aldehydes acrolein, 3-aminopropanal (3-AP), 3-acetamidopropanal (3-AAP) and 4-aminobutanal (4-AB)

3-AP sources

$NH_2–(CH_2)_3–NH–(CH_2)_4–NH–(CH_2)_3–NH_2$ (SPM) ⇒ SPD + H_2O_2 + $NH_2–CH_2–CH_2–CHO$

$NH_2–(CH_2)_3–NH–(CH_2)_4–NH_2$ (SPD) ⇒ PUT + H_2O_2 + $NH_2–CH_2–CH_2–CHO$

$NH_2–(CH_2)_3–NH_2$ (PDA) ⇒ H_2O_2 + NH_3 + $NH_2–CH_2–CH_2–CHO$

3-AAP sources

N-acetyl-SPM ⇒ SPD + H_2O_2 + $CH_3–CO–NH–CH_2–CH_2–CHO$

N-acetyl-SPD ⇒ PUT + H_2O_2 + $CH_3–CO–NH–CH_2–CH_2–CHO$

4-AB sources

$NH_2–CH2–CH_2–CH_2–CH_2–NH_2$ (PUT) ⇒ H_2O_2 + $NH_2–CH2–CH_2–CH_2–CHO$

N-acetyl-PUT ⇒ H_2O_2 + $NH_2–CH2–CH_2–CH_2–CHO$

SPD ⇒ PDA + H_2O_2 + $NH_2–CH2–CH_2–CH_2–CHO$

Acrolein sources

SPM ⇒ SPM aldehyde ⇒ SPD + H_2O_2 + $CH_2=CH–CHO$

SPM ⇒ SPM dialdehyde ⇒ PUT + H_2O_2 + $CH_2=CH–CHO$

SPD ⇒ SPD aldehyde ⇒ PUT + H_2O_2 + $CH_2=CH–CHO$

SPD, spermidine; SPM, spermine; PUT, putrescine; PDA, 1,3-propanediamine. Metabolism involves the FAD-dependent amine oxidases (EC 1.4.3.4) monoamine oxidase (MAO), spermine oxidase, and polyamine oxidase (PAO) and the copper-dependent amine oxidases (EC 1.4.3.6) diamine oxidase (DAO) and semicarbazide-sensitive amine oxidase (SSAO).

2. Results

2.1. *Aldehyde cytotoxicity*

The rat retinal cell line, E1A-NR.3 (Fig. 1) was most sensitive to acrolein toxicity, followed closely by hydrogen peroxide. These data agree with previous reports of direct mitochondrial toxicity with acrolein (Picklo and Montine, 2001; Pocernich and Butterfield, 2003) and hydrogen peroxide (Hoyt et al., 1997). The concentration–response curves for 24-h LDH release with 4-aminobutanal were almost identical to those of 3-AP (Fig. 1); consistent with a similar pK_a (3-AP = 9.3; 4-AB = 9.8), which favors intralysosomal accumulation of both 3-AP (Li et al., 2003) and 4-AB since the lysosomal pH is around 4.5. In contrast, 3-AAP in the same concentration range was not cytotoxic (Fig. 1). 3-AAP is a degradation product of acetylated polyamines that accumulate as a result of augmented spermidine/spermine N-acetyltransferase activity. While 3-AAP has the aldehyde function of 3-AP, it lacks the free amino group (Table 1). Previous work has demonstrated that raising lysosomal pH with NH_4Cl decreases the lysosomal ion trapping of 3-AP and dramatically reduces the cytotoxicity of this aminoaldehyde (Li et al., 2003; Yu et al., 2003). Our data are the first to demonstrate that acetylation of the amino group also limits the ability of 3-AAP to accumulate in lysosomes and initiate a cytotoxic cascade. This is probably a result of steric hindrance limiting access to a transporter and not the result of alterations in pK_a since 3-AAP has a higher pK_a (15.8) than 3-AP (9.3).

These data also suggest that reaction of the aldehyde function of 3-AP and 3-AAP with sulfhydral and amino groups of structural proteins or enzymes outside of the lysosomal compartment is probably not a significant component of the toxicity of 3-AP. Our data would also suggest that 3-AAP is not converted to 3-AP or acrolein under our culture conditions. While there is an inconsistent literature suggesting that 3-AP and 3-AAP can spontaneously degrade to acrolein in some conditions (reviewed by Seiler, 2000) this was unlikely in our cultures based on the potent toxicity of acrolein but lack of toxicity of 3-AAP up to 1 mM (Fig. 1).

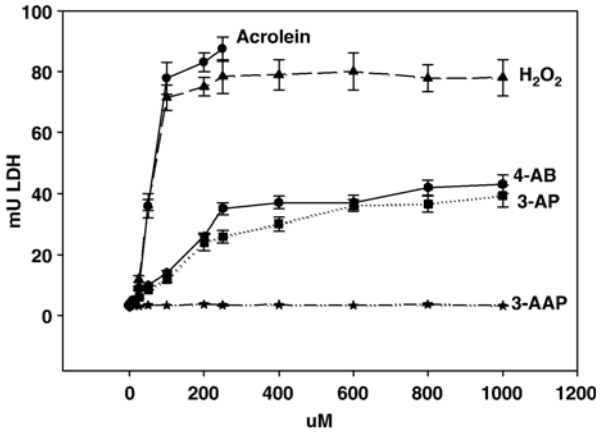

Fig. 1 – Concentration–response for aldehyde and hydrogen peroxide neurotoxicity in rat retinal ganglion cell cultures (E1A-NR.3). 4-AB, 4-aminobutanal; 3-AP, 3-aminopropanal; 3-AAP, 3-acetamidopropanal. 24-h media LDH levels. Mean ± SEM (n = 6 wells). This experiment was repeated 2 times.

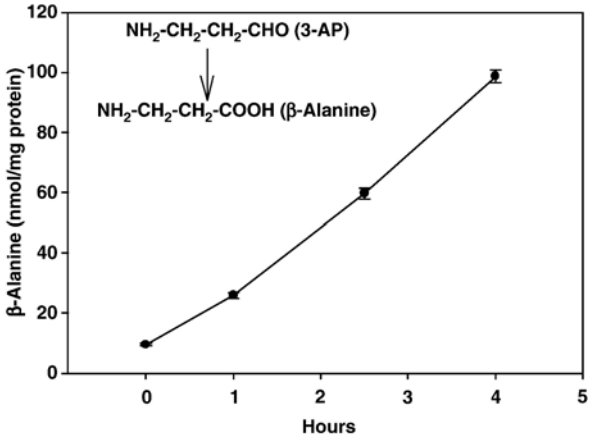

Fig. 2 – Time course of β-alanine accumulation in retinal ganglion cells after addition of the reactive aldehyde 3-AP (200 μM) to the medium. Mean ± SEM (n = 6 wells). Linear regression of β-alanine levels at 1, 2.5 and 4 h yielded an accumulation rate of 24.3 ± 1.0 nmol/mg protein/h (r = 0.999). This experiment was repeated 2 times.

2.2. *3-AP metabolism*

Previous studies of acrolein and 4-hydroxynonenal have shown that these aldehydes are mainly metabolized (Srivastava et al., 2001; Meyer et al., 2004) to their corresponding acids by aldehyde dehydrogenases (ALDH) and to mercapturic acids via conjugation with glutathione (glutathione-S-transferases, GST). GST activity is dependent upon an electrophilic center in the substrate and since aminoaldehydes lack such a center, we speculated that ALDH may play a greater role in the metabolism of 3-AP. Monitoring β-alanine production, the ALDH product of 3-AP, we found a time-dependent linear increase in cellular β-alanine levels in retinal ganglion cells with a rate constant of 24.3 ± 1 nmol/mg protein/h (Fig. 2). In contrast, incubation of retinal ganglion cells with 50 μM acrolein for 4 h did not alter β-alanine levels (data not shown), indicating that increases in this amino acid were not the result of cellular stress induced by aldehyde load but due to 3-AP metabolism.

3. Discussion

Aminoaldehydes (3-AP and 4-AB), 3-AAP, acrolein and hydrogen peroxide are all products of polyamine degradation (Table 1). The 2-alkenals (e.g. acrolein), 4-hydroxy-2-alkenals (e.g., 4-hydroxy-nonenal) and ketoaldehydes (e.g., malondialdehyde) activate the intrinsic apoptotic cascade, independent of lysosomes (Kruman et al., 1997; Picklo and Montine, 2001; Pocernich and Butterfield, 2003; Zarkovic, 2003). In contrast, aminoaldehydes concentrate in lysosomes by proton-trapping where they initiate leakage of proteolytic enzymes via lysosomal rupture, thereby compromising mitochondrial

integrity (Brunk et al., 2001; Li et al., 2003; Yu et al., 2003, 2004). While hydrogen peroxide may act in synergy with reactive aldehydes *in vivo*, cell culture studies of augmented polyamine metabolism and *in vivo* neuroprotection with aldehyde sequestering agents, indicate that reactive aldehydes are the major cytotoxic agents and that hydrogen peroxide is not a significant mediator of apoptosis or cell death (Ivanova et al., 1998; Seiler, 2000; Wood et al., 2006b). Hydrogen peroxide toxicity is probably limited by the large reserves of antioxidative defense mechanisms that include substantial catalase activities in glia and microglia (Dringen, 2005). While these are valuable data, they cannot rule out a potential synergistic action of hydrogen peroxide in complex pathological cascades *in vivo*.

Evidence continues to accumulate for the role of reactive aldehydes in neuronal cell death in a number of neurological disorders. This includes the accumulation of toxic aldehydes in Alzheimer's disease and mild cognitive impairment (Ahmed et al., 2005; Calingasan et al., 1999; Choei et al., 2004; Lovell et al., 2001; Montine and Morrow, 2005; Sayre et al., 1997; Williams et al., 2006), Parkinson's disease (Ilic et al., 1999; Selley, 1998), and multiple sclerosis (Hunter et al., 1985; Calabrese et al., 1994; Bizzozero et al., 2005). These neurodegenerative diseases are characterized by increases in polyamine synthesis and metabolism (Bernstein and Muller, 1995; Xu et al., 2003). These increases in polyamine metabolism result in the generation of hydrogen peroxide, acrolein, aminoaldehydes (3-AP and 4-AB) and acetamidopropanal (Table 1). Previous workers have demonstrated that acrolein is directly toxic to mitochondria while the aminoaldehyde 3-AP accumulates in lysosomes resulting in leakage of proteases that compromise mitochondrial function. Our data are the first to undertake a comparison of the concentration–response relationships for the toxicity of polyamine metabolites and allow the following conclusions:

1. 4-Aminobutanal (4-AB), an aminoaldehyde metabolite of polyamine metabolism like 3-AP, is cytotoxic. This aminoaldehyde would be predicted to be lysomotropic and cytotoxic but this is the first report to directly demonstrate its toxicity.
2. Acetamidopropanal (3-AAP), the *in vivo* product of acetylated polyamines is not cytotoxic. Again, loss of the free amine group would predict such a loss of cytotoxicity; however, this is the first data to directly validate that prediction. This is an important finding in that significant increases in 3-AAP can occur in vivo with activated polyamine metabolism (Seiler, 1995, 2000, 2004).
3. 3-AAP is not metabolized to 3-AP or acrolein to a significant extent since millimolar concentrations are not cytotoxic.
4. Metabolism of 3-AP by aldehyde dehydrogenase to its corresponding acid, β-alanine, was demonstrated in E1A-NR.3 retinal ganglion cells to proceed at a rate of 24.3± 1 nmol/mg protein/h. Since 3-AP is not metabolized by glutathione-S-transferases, this is presumably the major inactivation pathway of aminoaldehydes. The disposition of aminoaldehydes in a cell culture system is complex in that the largest proportion of the aldehyde binds to serum proteins in the media (Wood et al., 2006b). Additionally, binding to cell membranes, cellular proteins, cellular organelles and sequestration by lysosomes are all routes of aminoaldehyde disposition. In contrast, in vivo aminoaldehydes are mainly generated intracellularly and not secreted (Seiler, 1995, 2000, 2004).

In summary, our data support the hypothesis that aminoaldehyde neurotoxicity is mediated via accumulation in lysosomes (Brunk et al., 2001; Li et al., 2003; Yu et al., 2003, 2004) and that proteases released by ruptured lysosomes are responsible for subsequent loss of mitochondrial function (Wilson et al., 1987; Kagedal et al., 2001; Zhao et al., 2003). Increased lysosomal fragility has been suggested to be responsible for neuronal losses after stroke (Ivanova et al., 1998, 2002), for neuronal and white matter lesions in Alzheimer's disease (Nixon and Cataldo, 2006; Kobayashi et al., 2002) and for lesions in multiple sclerosis (McKeown and Allen, 1979). Hence, "aldehyde load" may play a pivotal role in a number of neurodegenerative diseases. This hypothesis can be effectively evaluated by the preclinical and clinical evaluation of aldehyde sequestering agents (Burcham et al., 2002; Hipkiss, 2001; Ivanova et al., 2002; Wood et al., 2006b, 2006c).

4. Experimental procedures

4.1. *Materials*

Dulbecco's minimal essential medium (DMEM) was purchased from GIBCO, Long Island, NY. Fetal bovine serum (FBS) was from Hyclone, Logan, UT. [$^2H_4$]β-alanine was purchased from Cambridge Isotopes Labs (Woburn, MA). The Cytotoxicity Detection Kit (LDH) was obtained from Roche Applied Science, Indianapolis, IN. The protein BCA kit was purchased from Pierce, Rockford, IL and 3-aminopropanal diethyl acetal from TCI America, Portland, OR. All other reagents were purchased from Sigma Chemicals, St. Louis, MO. The 1.5 ml screw top microfuge tubes were purchased from Sarstedt, Newton, SC.

4.2. *Aldehyde syntheses*

3-AP was synthesized from 3-aminopropanal diethyl acetal as described previously (Wood et al., 2006b). For the synthesis of 3-AAP, 1.03 ml of acetic anhydride was added over 25 min to a stirred solution of amino-3,3-diethoxypropane (1.61 ml) in 10 ml of dry pyridine at 0 °C. After 30 min of stirring the reaction mixture was warmed to room temperature and stirred for 24 h. The pyridine, acetic acid and acetic anhydride were removed in a rotary evaporator under high vacuum at 50–60 °C, yielding acetamido-3,3-diethoxypropane. Acetamidopropanal (3-AAP) was prepared by hydrolysis of acetamido-3,3-diethoxypropane in 1.5 M of hydrochloric acid with 30 min of stirring at room temperature. 3-AAP was concentrated in a Savant concentrator, quantitated with the Purpald reaction (Wood et al., 2006b) and characterized by NMR and GC-MS. For GC-MS, 3-AAP was derivatized with pentafluorobenzyl hydroxylamine (Wood et al., 2006b) resulting in a 310 Da derivative. The HP-5 capillary column (25 m, 0.25 mm i.d. and 0.25 μm thickness) was held at 100 °C for 1 min followed by 30 °C gradient to 300 °C, with carrier gas (He) flow of 1.2 ml/min.

The reagent gas was ammonia. In PCI, 311 $[MH]^+$ and 328 $[M+18]^+$ were the dominant ions while in NCI, 290 $[M\text{-}HF]^-$ was the dominant ion; with a retention time of 6.2 min.

For the synthesis of 4-aminobutanal (4-AB), 1.03 ml acetic anhydride were added to a stirred solution of 1-amino-4,4-diethoxybutane (1.79 ml in 10 ml of dry pyridine) at 0 °C. After 30 min stirring at 0 °C, the reaction mixture was warmed to room temperature and stirred for an additional 24 h. Progress of the reaction was monitored by TLC. The pyridine, acetic acid and acetic anhydride were removed in a rotary evaporator under high vacuum at 50–60 °C yielding 1-acetamido-4,4-diethoxybutane. 4-AB was prepared by hydrolysis of 1-acetamido-4,4-diethoxybutane in 1.5 M HCl with 30 min of stirring at room temperature. 4-AB was concentrated in a Savant concentrator, characterized by GC-MS and quantitated with the Purpald reaction. For GC-MS 4-AB was reacted with pentafluorobenzyl hydroxylamine and *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluroacetamide containing 1% tert-butyldimethylchlorosilane (Wood et al., 2006b), resulting in a 396 Da derivative. The dominant ions were 397 $[MH]^+$ and 414 $[M+18]^+$ in PCI and 356 $[M\text{-}2HF]^-$ in NCI with a retention time of 7.8 min.

4.3. *Cell cultures and sample preparation*

The rat retinal cell line, E1A-NR.3 (Seigel et al., 2004; Wood et al., 2006b) was grown in DMEM, containing 10% FBS, in 75 cm^2 flasks. For neurotoxicity assays, cells were plated in 48-well tissue culture plates and exposed to aldehydes or H_2O_2 for 24 h. Media was collected and assayed for LDH using the Roche assay kit. Rabbit muscle LDH was used for the standard curve. All aldehydes were dissolved in PBS for addition to cultures.

For 3-AP metabolism studies, 200 μM 3-AP was added to retinal ganglion cell cultures in 12-well plates. At 1, 2.5 and 4 h the media was discarded and the wells washed with 2 ml of PBS before addition of 750 μl of cold 1 N HCl containing 2 nmol of $[^2H_4]$β-alanine as internal standard. The plates were scraped and the well contents transferred to 1.5 ml microfuge tubes. After sonication, the samples were centrifuged at 25,000×*g* for 40 min at 4 °C and the supernatants transferred to clean microtubes before being dried overnight in a Savant concentrator.

4.4. *GC-MS analyses of β-alanine*

For the analyses of β-alanine, the dried samples were derivatized with tBDMS in acetonitrile (Wood et al., 2006a). Aliquots of 1 μl were injected splitless. The HP-5 capillary column (25 m, 0.25 mm i.d. and 0.25 μm thickness) was held at 100 °C for 1 min followed by 30 °C gradient to 300 °C, with carrier gas (He) flow of 1.2 ml/min. Selected ion monitoring was in PCI with ammonia as the reagent gas. The $[MH]^+$ ions monitored at 5.2 min. were 318 for β-alanine and 322 for $[^2H_4]$β-alanine.

4.5. *Proteins*

The 25,000×*g* protein pellets were solubilized in 200 μl of 0.5 N NaOH and the protein content assayed via the BCA procedure. For the metabolism studies, the average protein content per well of a 12-well plate was 50 μg for the retinal ganglion cells.

4.6. *Statistical analysis*

All data are presented as mean±SEM for groups of 6 tissue culture wells. Data were analyzed by one-way ANOVA followed by the Dunnett's *t* test for comparisons to control.

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