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Gαq Reduces cAMP Production by Decreasing Gas Protein Abundance

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Abstract

The heterotrimeric guanine nucleotide-binding protein Gαq transduces signals from heptahelical transmembrane receptors (e.g., α₁-adrenergic, endothelin 1A, and angiotensin II) to stimulate generation of inositol-1,4,5-trisphosphate and diacylglycerol. In addition, Gαq decreases cAMP production, through unknown mechanisms, and thus affects physiological responsiveness of cardiac myocytes and other cells. Here, we provide evidence that Gαq expression increases Gas ubiquitination, decreases Gas protein content, and impairs basal and β₁-adrenergic receptor-stimulated cAMP production. These biochemical and functional changes are associated with Akt activation. Expression of constitutively active Akt also decreases Gas protein content and inhibits basal and β₁-adrenergic receptor-stimulated cAMP production. Akt knockdown inhibits Gαq-induced reduction of Gas protein. In addition, MDM2, an E3 ubiquitin ligase, binds Gas and promotes its degradation. Therefore, increased expression of Gαq decreases cAMP production through Akt-mediated Gas protein ubiquitination and proteasomal degradation.

Keywords

G protein; ubiquitination; proteasomal degradation; Akt; cAMP

INTRODUCTION

Heptahelical transmembrane receptors comprise the largest and most versatile receptor family in the mammalian genome and are ubiquitously expressed. Heterotrimeric guanine nucleotide-binding proteins (G proteins) are pivotal mediators of signaling pathways initiated by these receptors. Each G protein selectively binds to specific receptors and transduces the signal to a specific set of downstream effectors [1]. For instance, Gas transduces signals from the β₁-adrenergic receptor (β₁AR) to stimulate cAMP production by adenylyl cyclase, and Gαq transduces signals from the α₁-adrenergic receptor (α₁AR) to stimulate inositol-1,4,5-trisphosphate and diacylglycerol generation *via* phospholipase C. These signals, in turn, activate additional downstream effectors, such as protein kinases, to regulate a diverse range of biological functions [1].

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In addition to mediating these classical linear signaling pathways, a specific heptahelical G protein-coupled receptor may regulate signaling pathways activated by another receptor. This interaction has been referred to as “cross-talk” between two signaling pathways. One typical example of cross-talk is inhibition of β_1 AR-mediated cAMP production by α_1 AR stimulation in the heart [2]. This inhibition of cAMP production is associated with depressed left ventricular (LV) contractility *in vivo* [2]. Similarly, cardiac-directed expression of Gαq decreases β AR-mediated cAMP production and impairs LV function [3;4;5]. These examples underscore the potential importance of Gαq on β_1 AR-mediated cAMP production, and mandate studies to determine mechanisms underlying these interactions. Previous reports indicate that inhibition of β AR-mediated cAMP production by Gαq activation does not occur via receptor desensitization or reduced adenylyl cyclase expression [3;4;6]. In the current study, we tested the hypothesis that Gαq activation inhibits β_1 AR-mediated cAMP production at the Gas level. Our results reveal a novel mechanism by which Gαq influences Gas signaling by regulating Gas protein levels.

MATERIALS AND METHODS

Animals

Transgenic mice with cardiac-directed expression of Gαq (initial transgenic mouse line provided by Dr. G.W. Dorn, University of Cincinnati) were used. Left ventricles from 10-week-old Gαq mice (Gαq) and transgene negative littermates (CON) were snap frozen with liquid nitrogen, and stored at -80°C . This study was approved by Animal Use and Care Committee of VA San Diego Healthcare System, in accordance with AAALAC guidelines.

Cell culture

Cardiac myocytes and fibroblasts were isolated from 1-day-old Sprague-Dawley rats by Percoll centrifugation as previously described [7]. HEK293 cells were from ATCC. Cells were maintained in DMEM supplemented with 10% fetal bovine serum in a humidified incubator with 5% CO_2 at 37°C . DNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. Forty-eight hours after transfection, cells were harvested for Western blotting.

Reagents

Protease inhibitors MG132 (carbobenzoyloxy-L-leucinyll-L-leucinyll-L-leucinal), lactacystin, ALLM (*N*-acetyl-L-leucinyll-L-leucinyll-methioninal), MDL (carbobenzoxyl-valyl-phenylalaninal), and E64 (*trans*-epoxysuccinyll-L-leucylamido(4-guanidino)butane) were from Calbiochem. Calcium activators ionomycin and thapsigargin, PKC activator phorbol 12-myristate 13-acetate (PMA), and PKC inhibitors bisindolylmaleimide (BIM) and Et-8-OCH₃ were also from Calbiochem. ON-TARGETplus SMARTpool small interfering RNA (siRNA) specific for human Akt1 was obtained from Dharmacon.

Antibodies to Gas(sc-383), Gαq(sc-393), and ubiquitin(sc-8017) were obtained from Santa Cruz Biotechnology; antibody to MDM2(clone SMP14) was purchased from BD Biosciences; antibodies to phosphor-Akt(S473)(#4051), Akt(#9272), and phosphor-MDM2(S166)(#3521) were obtained from Cell Signaling Technology; and antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; RDI-TRK5G4-6C5) was obtained from Research Diagnostic Inc.

Mammalian expression vectors encoding MDM2 (provided by Dr. J. Chen, University of South Florida) and Gαq (pcDNA-Gαq, University of Missouri-Rolla cDNA Resource Center) were used. Full length Gαq cDNA was subcloned adenovirus shuttle vector pE1Z for generating adenovirus encoding Gαq (Ad.Gαq). Empty adenovirus vector (Ad.NULL) was also

constructed. Adenovirus encoding constitutively active Akt (Ad.Akt) (provided by Dr. K. Walsh, Boston University) was expanded and purified.

Western blotting

LV samples or cultured cells were homogenized and Western blotting was performed as described previously [6]. Equal loading of samples and even transfer efficiency were monitored by reprobing stripped blots with GAPDH antibody. Quantification of protein content was performed using Gel-Pro[®] Analyzer (Media Cybernetics) as previously described [6].

Co-immunoprecipitation

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) in the presence of protein inhibitor cocktail (Roche). Cell lysates (500 µg total protein) were clarified by centrifugation, precleared by incubation with protein A/G beads (Pierce), and incubated for 5h at 4°C with immunoprecipitation antibodies. The resulting immune complexes were precipitated with protein A/G beads (Pierce), washed with RIPA buffer, and subjected to Western blotting.

Northern blotting

Total cellular RNA was extracted from LV samples using RNA STAT-60 (Tel-Test Inc.). Twenty micrograms of denatured total RNA was fractionated in a formaldehyde-agarose gel, and transferred to a Nytran membrane (Schleicher & Schuell), and hybridized with a ³²P-labeled murin *Gas* cDNA probe amplified by PCR using primer pairs: 5'-CGTGATGAACCTGCCGAAC-3' and 5'-CCTCCTGCAGGCGGTTAG-3'. Equal loading of samples and even transfer efficiency were monitored by re-probing stripped blots with β-actin probe. Quantification of mRNA expression was performed using Gel-Pro[®] Analyzer.

Akt knockdown by siRNA transfection

HEK293 cells grown to 50–60% confluency were transfected with 200 pmol ON-TARGETplus SMARTpool siRNA specific for human Akt1 using the X-tremeGENE siRNA transfection (Roche) reagent according to the manufacturer's instruction. Twenty-four hours after siRNA transfection, Ad.NULL or Ad.Gaq were added to the cell culture. Cells were harvested 24 h after viral infection and cell homogenates were used for Western blotting.

Statistical analysis

Data represent mean±SE; group differences were detected using one-way ANOVA, followed by Bonferroni *t*-testing. The null hypothesis was rejected when *p*<0.05.

RESULTS

Gaq expression decreases *Gas* protein but not mRNA content

To test the hypothesis that Gaq decreases βAR-mediated cAMP production at the *Gas* level, we first compared *Gas* protein content in LV samples from transgenic Gaq mice and CON mice by Western blotting. *Gas* protein content in LV homogenates from Gaq mice was reduced by 98% (CON: 666±23 densitometric units (du), Gaq: 16±1 du, *p*<0.0001, *n*=8; Fig. 1A). Despite such a marked decrease in *Gas* protein content, Northern blotting showed no difference in contents of *Gas* mRNA in CON and Gaq mouse hearts (CON: 3147±45 du, Gaq: 3208±116 du, *p*=0.6, *n*=6; Fig. 1B). These results indicate that Gaq expression affects *Gas* protein content post-transcriptionally.

We then compared contents of other proteins in LV samples from CON and Gaq mouse hearts. Western blotting showed no change in GAPDH and calsequestrin protein levels after Gaq

expression (Fig. 1C). Content of phospholamban protein, in contrast to Gas, was increased by Gαq expression (CON: 611±88 du, Gαq: 1532±169 du, $p<0.001$, $n=6$; Figure 1C), indicating that Gαq expression does not result in a general diminution in protein levels in mouse hearts.

To determine whether the effect of Gαq expression was cell type-dependent, we isolated cardiac myocytes and cardiac fibroblasts, and infected these cells with Ad.Gαq (4 pfu/cell). Western blotting showed that Gas protein content was decreased by Gαq expression in both cardiac myocytes and fibroblasts (Fig. 1D), indicating that reduction in Gas conferred by increased Gαq expression was not cell type-dependent.

To determine whether Gas down-regulation by Gαq also occurs in cells not derived from the heart, we measured Gas protein content in HEK293 cells transfected with pcDNA-Gαq. Western blotting showed that Gαq expression reduced Gas protein content in HEK293 cells (Fig. 1E), as it did in cardiac myocytes and fibroblasts and *in vivo*. We also found that Gαq expression did not affect Gas mRNA content by Northern blotting in HEK293 cells (Fig. 1E). In addition, a dose-response relationship between increased Ad.Gαq amount (0.5–8 pfu/cell) and reduction in Gas protein content (0–74% reduction) was documented (Fig. 1F). These results indicate that Gαq down-regulates Gas protein content post-transcriptionally in multiple cell types and in proportion to the Gαq expression level.

Gαq decreases Gas protein by facilitating proteasomal degradation

To gain insight into how Gαq expression decreases Gas protein content, we infected HEK293 cells with Ad.Gαq and treated these cells with protease inhibitors. Western blotting showed that MG132 and lactacystin, two specific proteasomal inhibitors, blocked Gαq-induced reduction of Gas protein. In contrast, Gas protein content was not altered by calpain inhibitor ALLM, and was marginally increased by lysosomal protease inhibitors MDL and E64 (Fig. 2A), indicating that Gαq-induced increased Gas turnover is mediated by proteasomal degradation.

We then treated isolated cardiac myocytes and cardiac fibroblasts with MG132, ALLM, MDL, and E64, and measured Gas protein content. Western blotting showed that only the proteasomal inhibitor MG132 blocked degradation of Gas protein in both cardiac myocytes and cardiac fibroblasts (Fig. 2B), confirming that Gas protein turnover in these cardiac-derived cells is also mediated by proteasomal degradation.

Gαq increases Gas ubiquitination

Proteins are usually first marked by conjugation with ubiquitin before being targeted for proteasomal degradation. To determine whether Gas underwent ubiquitination, Gas was immunoprecipitated from HEK293 cell homogenates, and ubiquitin-conjugated Gas was determined by Western blotting using an antibody against ubiquitin. A smear of ubiquitin-conjugated Gas was noted in HEK293 cell homogenates, and accumulation of polyubiquitinated Gas was found in cells incubated with MG132 (Fig. 2C). Infection of HEK293 cells with Ad.Gαq increased the density of the smear of ubiquitin-conjugated Gas, both with and without MG132 incubation (Fig. 2C). These data indicate that Gas is subject to polyubiquitination in intact cells, and expression of Gαq increases the amount of polyubiquitinated Gas.

Akt activation decreases Gas protein content and function

To determine the mechanism by which Gαq facilitates Gas proteasomal degradation, we first examined the potential role of Ca^{2+} and PKC (both activated by Gαq). Treatment with the Ca^{2+} activators ionomycin and thapsigargin, and the PKC activator PMA did not alter Gas protein content (Fig. 3A). In addition, treatment with the PKC inhibitors BIM and Et-8-

OCH₃ did not inhibit Gαq-induced Gas degradation (Fig. 3A), suggesting that Gαq-facilitated Gas proteasomal degradation is not mediated by Ca²⁺ or PKC activation.

We then investigated whether Gαq-facilitated Gas protein degradation was mediated by Akt, another mediator for Gαq signaling [8]. In HEK293 cells, we found that Gαq increased protein content of Ser473-phosphorylated Akt, a surrogate measurement for Akt activation (Fig. 3B). In addition, inclusion of the PI3K inhibitors, LY294002 and wortmannin, inhibited Gαq-induced Akt activation. Furthermore, similar to expression of a constitutively active form of Akt, expression of Gαq increased MDM2 phosphorylation at Ser166, a preferred site for Akt-mediated phosphorylation (Fig. 3C). Increased MDM2 phosphorylation also was observed in LV samples from mice with cardiac-directed Gαq expression (Fig. 3D). These results confirm that Gαq expression activates Akt.

We then explored whether activation of Akt could affect Gas protein content and function. Expression of the constitutively active form of Akt decreased Gas protein content, and this decrease was blocked by the proteasomal protein degradation inhibitor MG132 (Fig. 3C). We also found that expression of a constitutively active form of Akt, like expression of Gαq, increased ubiquitinated Gas protein content (Fig. 3E), a necessary step for facilitated proteasomal degradation. In addition, expression of Gαq and constitutively active Akt not only decreased basal cAMP production (Ad.Gαq vs Ad.NULL: 88% reduction, $p < 0.001$; Ad.Akt vs Ad.NULL: 89% reduction, $p < 0.001$), but also decreased isoproterenol-stimulated cAMP production in isolated cardiac fibroblasts (Ad.Gαq vs Ad.NULL: 72% reduction, $p < 0.001$; Ad.Akt vs Ad.NULL: 73% reduction, $p < 0.001$; Fig. 3F). Finally, knockdown of Akt1 in HEK293 cells using siRNA inhibited Gαq-induced reduction of Gas protein (Fig. 3G). These data indicate that Akt activation mediates Gαq-facilitated Gas protein turnover and function.

MDM2 expression decreases Gas protein content

To explore whether MDM2, an E3 ubiquitin ligase, influences Gas protein degradation, we first tested whether MDM2 was physically associated with Gas. We found that Gas was co-immunoprecipitated by a specific anti-MDM2 antibody from lysate from HEK293 cells expressing Gαq (Fig. 4A). This physical association of Gas and MDM2 was confirmed by co-immunoprecipitation of the Gas-MDM2 complex using anti-Gas antibody and immunoblotting with anti-MDM2 antibody (Fig. 4A). Incubation of HEK293 cells with the proteasomal degradation inhibitor MG132 increased the amount of co-immunoprecipitated Gas and MDM2 (Fig. 4A). We then examined whether MDM2 affected Gas protein content. Western blotting showed that increased MDM2 expression decreased Gas protein content in HEK293 cells (Fig. 4B). Taken together, these data indicate that MDM2 binds to Gas and has a direct impact on Gas protein content.

DISCUSSION

In this study, we identified a novel mechanism by which increased Gαq influences the Gas-mediated signaling pathway. We found that Gαq expression increased Gas ubiquitination, decreased Gas protein content, and impaired basal and β₁AR-stimulated cAMP production. These biochemical and functional changes were associated with Akt activation. We demonstrated that expression of constitutively active Akt decreased Gas protein content and inhibited basal and β₁AR-stimulated cAMP production, and Akt knockdown inhibited Gαq-induced reduction of Gas protein. In addition, we provided evidence that MDM2, an E3 ubiquitin ligase, bound Gas and promoted Gas protein degradation.

Heterotrimeric G proteins play a major role in transducing signals from hundreds of cell membrane G protein-coupled receptors [1]. Recent studies also describe accessory proteins as new Ga binding partners, which provide additional signal input independent of G protein-

coupled receptors and introduce additional functional roles for G proteins [9]. The physiological and pathophysiological responses that are directly regulated by G proteins are diverse, ranging from embryonic development to clinical heart disease and cancer. The functional specificity of G proteins is largely determined by the α subunit, although $\beta\gamma$ subunits also play important roles [10]. Although cross-talk between G_{aq} and G_{as} signaling pathways has been proposed [11;12], those studies only measured transient changes of cAMP production in one cell type *in vitro*. The effect of long-term G_{aq} activation on G_{as} signaling was established in these studies. Our data provide direct evidence that G_{aq} reduces G_{as} signaling by facilitating G_{as} protein proteasomal degradation in multiple cells and also in intact hearts.

The ubiquitin-proteasome system plays an essential role in numerous biological and pathological processes [13]. Gpa1, an α subunit of a G protein in *Saccharomyces cerevisiae*, appears to undergo ubiquitination and proteasomal degradation [14], but whether this also occurs in mammalian G proteins is unknown. Recently it was shown that MG132 blocks mammalian G_{as} degradation [15], but mechanisms have not been established. Our data not only demonstrate that G_{aq} facilitates G_{as} proteasomal degradation and decreases cAMP production, but also provide evidence that Akt mediates this process.

Ubiquitination occurs in a three-step process catalyzed by ubiquitin-activating enzyme (E1), conjugating enzyme (E2), and ubiquitin ligase (E3) [13]. MDM2, an E3 ubiquitin ligase, not only interacts with tumor suppressor protein p53 and other nuclear proteins and regulates their function, but also binds cytoplasmic β -arrestin [16]. Association of MDM2 and β -arrestin brings β_2AR , insulin-like growth factor receptor, and G protein-coupled receptor kinase 2 to the vicinity of MDM2. MDM2-catalyzed ubiquitination of these membrane or membrane-bound proteins is essential for their internalization or degradation [16;17;18]. In the present study, we showed that MDM2 interacted with G_{as} , and promoted G_{as} ubiquitination and proteasomal degradation. This binding of MDM2 and G_{as} , unlike that with β_2AR , insulin-like growth factor receptor, and G protein-coupled receptor kinase 2 [16;17;18], does not appear to require the presence of β -arrestin — we could not detect the G_{as}/β -arrestin complex by co-immunoprecipitation (data not shown), while the $G_{as}/MDM2$ complex was readily detectable under the same experimental conditions.

Protein ubiquitination and proteasomal degradation can be stimulated or inhibited by protein kinase activation [19]. Akt promotes proteasomal degradation of transcription factors, e.g. p53, FOXO, and NFAT, but inhibits protein turnover of itself and BRF1, a zinc finger protein with mRNA decay properties. Our data suggest a previously unrecognized intracellular signaling event in which Akt activation mediates G_{aq} -induced G_{as} ubiquitination and proteasomal degradation. Alternatively, increased G_{aq} may sequester $G\beta\gamma$ subunits away from G_{as} and subsequently promote selective ubiquitination and degradation of G_{as} . However, our findings in this study do not support this notion: (1) Akt expression, in the absence of G_{aq} overexpression, increased G_{as} ubiquitination and proteasomal degradation. (2) Akt knockdown inhibited G_{aq} -induced reduction of G_{as} . Since G_{as} contains a potential Akt phosphorylation motif LLRCRVLTSGI (consensus Akt phosphorylation motif: RXRXXS/T) surrounding threonine residue 190, it is possible that Akt promoted G_{as} degradation by increasing G_{as} phosphorylation, as shown in many other cases [19]. To exclude this possibility, we determined whether the synthesized LLRCRVLTSGI peptide is an Akt substrate. Using an *in vitro* phosphorylation assay with active recombinant Akt1, we found that this peptide is a poor Akt substrate (K_m : 123 μM , V_{max} : 27 nmol/min/mg) (unpublished data). It is therefore unlikely that direct G_{as} phosphorylation by Akt affects G_{as} protein stability. We are actively investigating whether Akt-promoted G_{as} degradation results from increased MDM2 E3 ligase activity via MDM2 stabilization [20].

Gαq transduces signals from heptahelical transmembrane receptors, e.g. α₁AR and angiotensin II receptor. Increased expression of Gαq leads to pathological cardiac hypertrophy and development of heart failure [3;5]. Reduced Gαq signaling (Gαq deletion or inhibition) appears to prevent maladaptive cardiac hypertrophy [21;22]. A polymorphism in the Gαq gene promoter region is associated with enhanced promoter activity, increased Gαq expression, higher prevalence of LV hypertrophy, accelerated mortality in heart failure [23;24]. Since cAMP is a critical regulator for LV contractile function, facilitated Gαs degradation and depressed cAMP production may represent a novel mechanism for Gαq-induced cardiac dysfunction.

In conclusion, our data reveal a novel mechanism by which Gαq decreases basal and βAR-stimulated cAMP production through Akt activation, increased Gαs ubiquitination, and facilitated Gαs proteasomal degradation.

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Fig. 1.

Cardiac-directed *Gαq* expression decreased *Gαs* protein, but not *Gαs* mRNA. (A) A representative Western blot showing decreased *Gαs* protein content in LV samples from 4 CON and 4 *Gαq* mice. (B) Northern blot showing normal *Gαs* mRNA content in LV samples from *Gαq* mice; β -actin was used as an internal control. (C) Western blots showing unchanged calsequestrin and GAPDH expression, and increased phospholamban (PLN) expression, in LV samples from *Gαq* mice. (D) Western blots showing decreased *Gαs* protein content in isolated cardiac myocytes and fibroblasts after gene transfer with Ad.*Gαq*. (E) *Gαs* protein was decreased in HEK293 cells transfected with p*Gαq* (vs empty plasmid vector pcDNA3.1), despite normal *Gαs* mRNA content. (F) Increased *Gαq* expression were associated with progressively reduced amounts of *Gαs*. GAPDH and β -actin were used as internal controls for Western and Northern blotting respectively.

Fig. 2.

Gαq expression increased Gas ubiquitination and proteasomal degradation. (A) In HEK293 cells, Gαq-induced reduction in Gas protein content was blocked by specific proteasomal inhibitors, MG132 (MG) and by lactacystin (LAC), but not by calpain inhibitor ALLM or lysosomal protease inhibitors MDL and E64. (B) In cardiac myocytes and fibroblasts, specific proteasomal inhibitor MG132, but not by calpain inhibitor ALLM or by lysosomal protease inhibitors MDL and E64, inhibited Gas protein reduction in cardiac myocytes and fibroblasts. (C) Increased Gαq expression increased Gas ubiquitination. Gas was immunoprecipitated from cell homogenates from parental HEK293 cells or HEK293 cells infected with Ad.NULL or Ad.Gαq. Ubiquitin conjugation was detected by Western blotting using a specific ubiquitin antibody.

Fig. 3.

Gαq-induced Gas protein degradation is mediated by Akt. (A) Gαq-induced reduction in Gas protein was not mediated by Ca²⁺ activators ionomycin (ION) and thapsigargin (THAP), PKC activator PMA, and PKC inhibitors BMI and Et-8-OCH₃ (ET8). (B) PI3K inhibitors LY294002 (LY) and wortmanin (WO) inhibited Gαq-induced Akt activation. MG, MG132. (C) Expression of Gαq and constitutively active Akt in HEK293 cells decreased Gas protein content and increased MDM2 phosphorylation. Treatment with the proteasomal inhibitor MG132 (MG) blocked the reduction of Gas protein content. (D) Cardiac-directed Gαq expression increased MDM2 phosphorylation in LV samples. (E) Expression of constitutively active Akt (Ad.Akt) increased Gas ubiquitination in HEK293 cells. Endogenous Gas-ubiquitin conjugate was co-immunoprecipitated by Gas antibody and detected by Western blotting using ubiquitin antibody. (F) Expression of Gαq and constitutively active Akt decreased basal and

isoproterenol (Iso)-stimulated cAMP production in isolated neonatal cardiac fibroblasts. Bars represent mean values from six experiments and error bars denote SEM (n=6). (G) Knockdown of Akt1 by siRNA blocked Gαq-induced reduction in Gαs protein.

A **B**

Fig. 4. MDM2 binds Gαs and decreases its content. (A) Co-immunoprecipitation of MDM2 and Gαs from cell homogenates from MG132-treated HEK293 cells infected with Ad.Gαq. MG, MG132. (B) Expression of MDM2 in HEK293 cells decreased Gαs protein content.