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Supplementation with brush border enzyme alkaline phosphatase slows aging

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Abstract

Diminished integrity of the intestinal epithelial barrier with advanced age is believed to contribute to aging-associated dysfunction and pathologies in animals. In mammals, diminished gut integrity contributes to inflammaging, the increase in inflammatory processes observed in old age. Recent work suggests that expression of intestinal alkaline phosphatase (IAP) plays a key role in maintaining gut integrity. IAP expression decreases with increasing age in mice and humans. Absence of IAP leads to liver inflammation and shortened lifespans in mice lacking the IAP gene. In normal mice, exogenous supplemental IAP reverses age-induced barrier dysfunction, improves aging-associated metabolic dysfunction, prevents microbiome dysbiosis (imbalance), and extends lifespan. Consistent with a IAP playing a conserved role in maintaining gut integrity, increased dietary IAP increases aging-diminished physical performance in flies. IAP helps maintain gut integrity in part by supporting the expression of tight junction proteins that maintain the intestinal epithelial barrier and by inactivating bacterial pro-inflammatory factors such as lipopolysaccharides (LPS) by dephosphorylation. Recombinant IAP is in late clinical trials for sepsis-associated acute kidney injury suggesting it may soon become available as a therapeutic. Taken together these reports support the idea that directly increasing IAP levels by supplemental recombinant IAP or by indirectly increasing IAP levels using dietary means to induce endogenous IAP may slow the development of aging-associated pathologies.

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0. Introduction.

Gut integrity, specifically the structural integrity and function of the intestinal epithelial barrier becomes impaired in older animals ranging from nematodes and fruit flies, to mice, monkeys and humans.[1 2 3 4 5] Normal gut functions include the adsorption of nutrients, ensuring that microbiota remain localized in the lumen of the intestine and protecting against microorganismal breaches by immune surveillance. Pathologies in humans associated with diminished intestinal barrier function include inflammatory bowel disease, ulcerative colitis, Crohn's disease, cancers, immune and neurological dysfunction[6]. It has been hypothesized that intestinal barrier dysfunction and alterations in microbiota contribute to inflammaging, the increase in aging-associated inflammation observed in many animals including humans[7 8 9]. Recent work suggests that alkaline phosphatases associated with the intestine play a key role in maintaining the intestinal barrier.[10 11]

Alkaline phosphatases are metalloenzymes that cleave phosphate from a variety of substrates. Humans express several isozymes classified as tissue-nonspecific (e.g. bone, liver, and kidney) and tissue-specific (e.g. intestine, placenta, and germ cells). Intestinal alkaline phosphatase (IAP) exhibits its biological activity in alkaline conditions with highest activity at pH 9.7. IAP expressed in the apical microvilli of the brush border of enterocytes plays an important role in gut homeostasis. IAP is expressed throughout the gastrointestinal tract with highest levels in the duodenum and lower levels in the jejunum, ileum, and colon. IAP is released into the gut lumen and can be detected in the stool[12].

A recent paper by Kuhn *et al.* demonstrates that IAP can slow the aging process by preserving gut barrier function and by reducing gut-mediated inflammaging[**10**]. Earlier human and animal data (reviewed in Fawley and Goulay[**13**]) demonstrated that exogenous IAP can provide a protective effect versus intestinal and systemic inflammation in a variety of diseases. In fact, recombinant human IAP is being evaluated in clinical trials. This new paper meticulously dissects the mechanism by which IAP supports gut health and thereby plays a critical role in aging.

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1. Age-dependent decline in IAP activity is associated with reduced gut barrier function and systemic inflammation.

Ileal IAP concentrations dropped by >50% from age 20 to age 80 in a cohort of 60 human volunteers. In mice, stool and ileal IAP concentrations decreased significantly (~40-50%) from young (4 month) to old individuals (21 month). These changes were associated with an increase in the "leakiness" of the murine gut measured by uptake of FITC-dextran. This breach of barrier function was associated with an uptick in serum endotoxin assumed to be the stimulus for increased systemic levels of inflammatory cytokines TNFalpha and IL6. To support a mechanism previously described by this group[14], local intestinal expression of tight junction proteins occludin and ZO-1 were observed to drop significantly suggesting that the integrity of the tight junctions was compromised. In each of these experiments the direction of the age-related changes (i.e. tight junction dysfunction-->gut leakiness-->endotoxemia-->cytokine levels-->inflammation) were augmented in specific IAP knock-out (KO) mice and supported to idea of administration of oral IAP as a "replacement therapy" for inflammaging[10].

2. Absence of IAP enhances aging-related liver inflammation and steatosis.

The portal circulation bathes the liver in gut-derived blood. Portal endotoxin more than doubled with aging and doubled again in the IAP knock-out mice. Levels of endotoxin measured by the Limulus Amebocyte Lysate (LAL) test were up to 1000-fold higher in the portal vs. systemic circulation. This was not unexpected that markers of liver inflammation such as TNFalpha and IL6 increased with aging and that the IAP knock-out mice exhibited even higher levels. These triggers of inflammation contributed to significant liver fat accumulation (steatosis) with age that was more severe in the IAP KO mice[10]. Such hallmarks of metabolic syndrome are characteristic of aging in many organisms.

3. **Exogenous, supplemental IAP reverses age-induced barrier dysfunction and extends lifespan.**

Based on earlier studies demonstrating a key role for IAP in gut dysfunction with aging[15], replacement therapy was next evaluated beginning at the age of 10 months, i.e. "middle-aged mice". Mice were supplied with autoclaved water containing bovine intestinal alkaline phosphatase (100 units/mL). The water bottles were refilled daily with 30ml (3000

units of IAP) or vehicle. Stool samples analyzed to document the increased intraluminal activity showed ~5-fold increase in IAP activity over baseline in the treated animals. Importantly, there were no differences in weight gain or food consumption among the various experimental groups of mice. Wild-type (WT) C57BL/6J mice lived a median of 23 months vs. the significantly shorter lifespan of the IAP-KO mice at 19 months. IAP supplemented WT mice exhibited a significantly longer median survival of 32 months[10].

The oral IAP supplementation reduced gut permeability as measured by FITC-dextran uptake and serum endotoxin levels. In addition, this replacement therapy reduced inflammatory cytokines IL6, IL1 beta, TNF alpha as well as fecal lipocalin, a marker of chronic intestinal inflammation.

4. IAP supplementation improves metabolic dysfunction associated with aging.

As noted above, IAP-KO mice exhibited the characteristic fatty liver of the metabolic syndrome observed in aging. In contrast the IAP-supplemented mice showed an improved metabolic profile with lower liver enzymes (AST, ALT), higher HDL cholesterol, lower triglycerides and LDL cholesterol and improved glucose tolerance.

The shorter or longer median mouse lifespans corresponding to lower or higher levels of intestinal IAP were also associated with increased (IAP-KO) or decreased (IAP supplemented) frailty scores[10].

5. IAP supplementation prevents aging-associated microbiome dysbiosis.

Sequential (prior to, 1 month, 6 months after treatment) fecal 16S ribosomal RNA sequencing and analysis was carried out to document age-associated changes in commensal bacterial populations of WT vs. IAP fed mice. Principal Coordinate Analysis (PCoA) of the relative abundance of bacterial phyla revealed significant changes during aging in the WT mice consistent with the literature in experimental models as well as humans. However, essentially no changes from the patterns found in the young mice were found among the IAP-treated animals as they aged[10].

6. IAP supplementation improves physical performance and survival of *Drosophilae.*

Drosophila are an important model organism for aging research due to their short lifespans and easy genetic manipulation. Despite the fact that the lineages of humans and Drosophilae separated some 700 million years ago, more than 50% of fly genes have homologs in the human genome[16]. Kuhn *et al.* carried out elegant studies of Drosophila showing that: 1) IAP activity decreases with age (young 3 weeks vs. old 7 weeks); 2) genetic knock-down of IAP reduces lifespan and this effect can be reversed with supplemental dietary IAP; 3) climbing activity of the flies (a measure of physical performance) is reduced during aging by 50% or more and can be reversed by dietary IAP[10].

7. Molecular mechanisms

IAP exhibits many important activities that sum together to maintain gut health. At the molecular level, Liu *et al.* showed that intestinal alkaline phosphatase gene deletion in mouse embryonic fibroblasts resulted in significantly lower levels of tight junction proteins ZO-1, ZO-2, and Occludin compared with levels in wild-type control cells[14]. Furthermore, IAP overexpression in colon cancer Caco-2 and T84 cells, used to model what happens in the normal human intestine, resulted in approximately 2-fold increases in the mRNA expression of ZO-1 and ZO-2[14]. IAP treatment ameliorated lipopolysaccharide-induced permeability in a Caco-2 trans-well system. Furthermore, IAP treatment preserved the localization of the ZO-1 and Occludin proteins during inflammation and was also associated with improved epithelial barrier function[10].

Gut lipopolysaccharide (LPS) plays a critical role in inflammaging[17]. Lumenal IAP has been documented to dephosphorylate LPS, which detoxifies LPS[18 19]. Kuhn *et al.* showed: 1) that systemic and portal endotoxin levels increase with aging; 2) that this increase stimulates many cytokine mediators of inflammation (e.g. IL 1 beta, IL6, TNF alpha) and 3) that oral supplementation with IAP results in reduction of endotoxin back to levels characteristic of young animals.

IAP can dephosphorylate a variety of other bacterial and host-derived ligands in addition to LPS Among these are CpG DNA, flagellin, UDP, etc., each of which works through a

specific receptor (e.g. toll-like receptors, PAMP, DAMPs, etc.) to exert its inflammatory impact on target cells[20 21]. In addition, Malo *et al.* demonstrated that IAP promotes the growth of beneficial gastrointestinal aerobic and anaerobic bacteria[22]. Compared with wild-type mice, IAP-knockout mice have more ATP in their GI luminal contents. Exogenous IAP promotes the growth of intestinal commensal bacteria by reducing the concentration of luminal ATP and other nucleotide triphosphates.

Medical implications

The therapeutic potential of alkaline phosphatase has previously been investigated in some detail. For example, two double-blind, randomized, placebo-controlled phase II clinical trials in septic patients with acute kidney injury (SA-AKI) demonstrated a beneficial renal effect of parenterally (i.v.) delivered bovine alkaline phosphatase[23 24 25]. Seeking to reduce possible immunogenicity, this Dutch group has developed a recombinant chimeric human alkaline phosphatase (huAP). Protective effects have been confirmed *in vitro* and *in vivo* in a rat model of LPS-induced AKI[26]. Safety and tolerability of the human enzyme was demonstrated in a phase I study[27] followed by the STOP-AKI trial, an international (53 recruiting sites), randomized, double-blind, placebo-controlled, dose-finding, adaptive phase 2a/2b study in 301 adult patients admitted to the intensive care unit with a diagnosis of SA-AKI[28]. After initial dose-range finding studies, patients were randomized to receive 1.6 mg/kg (n = 82) compared with placebo (n = 86). Among patients who were critically ill with SA-AKI, huAP phosphatase compared with placebo did not significantly improve short-term kidney function. However, Tang *et al.* (2020) carried out a systematic review and meta-analysis of the four randomized controlled trials involving huAP therapy of 392 patients with SA-AKI. In their analysis huAP showed a relatively late protective effect by improving endogenous creatinine clearance (ECC) at days 7, 14, and 28. ECC levels improved when patients received a huAP dose of 0.212 mg/kg. Mortality improved at days 28 and 90, respectively, when patients received a huAP dose of 1.6 mg/kg. Based on these studies, huAP is being evaluated in a phase III trial in SA-AKI sponsored by AM Pharma (AM-Pharma.com).

Rejuvenation Research
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IAP expression in the gut is reduced in patients with inflammatory bowel disease and exogenous administration of IAP has a beneficial therapeutic effect in rodent models of colitis (Tuin, 2009). Results from Phase I and Phase II clinical trials, with administration of bovine AP by duodenal drip, showed potential as a tolerable and efficacious treatment for ulcerative colitis. AM-Pharma conducted an open-label, multi-center trial, administering bovine AP continuously for 7 days. The trial recruited 21 moderate-to-severe UC patients who were unresponsive to treatment with steroids and immunosuppressants. The Mayo score showed a significant decrease in patients receiving bovine AP, versus baseline, at Day 21, and the MTWSI score at Day 21 and Day 63. In addition, the trial showed there were no clinically relevant adverse events causing withdrawal or considered serious, nor laboratory abnormalities nor antibody formation against the bovine AP. AM Pharma has developed an oral formulation of their recombinant huAP and plans additional human studies in inflammatory bowel disease.

The studies of Kuhn et al. support the replacement of IAP to support GI health and reduce several of the hallmarks of aging in humans. All studies described to date support the safety of both parenteral and oral administration of this important enzyme. Whether a less acute endpoint than SA-AKI or UC such a frailty can be addressed is certainly worth considering. Because AM-Pharma has raised a considerable amount of capital (~$200M) to support work to date one can assume that if/when their improved huAP is approved the cost will be substantial and unlikely to be affordable for long term chronic use.

It would be of interest to understand if IAP itself is the only alkaline phosphatase that can inactivate key gut disrupting substances such as LPS. Data indicate other alkaline phosphatases such as TNAP can inactivate LPS in blood for example[29]. Since TNAP is highly expressed in the liver, there may be benefits to diets that include liver, depending on the extent of active enzyme remaining. Diet may have profound effects on IAP expression: omega-3 fatty acids have been reported to increase IAP, as well as favoring microbiota that are not pro-inflammatory in mice[30]. Moreover, supplementation with probiotics that induce IAP or secrete alkaline phosphatase capable of inactivating LPS may be another strategy for maintaining gut integrity.

Of interest is that fasting or malnutrition lowers IAP expression and increases gut barrier dysfunction in mice and humans[31]. However, this seems contradictory to the beneficial effects observed with caloric restriction (CR), which is protective of intestinal barrier integrity[32] [33]. A possible explanation is that data suggest reducing calories alters the microbiome in rats and humans, favoring microorganisms that are less pro-inflammatory, thereby lowering the concentration of substances such as LPS. However, long-term data on IAP expression during CR is needed, as the decreases observed in short-term fasting may not be present over longer periods of time.

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Rejuvenation Research
Supplementation with brush border enzyme alkaline phosphatase slows aging (DOI: 10.1089/rej.2020.2335)

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