FEATURED IN THIS ISSUE:

- Novel Antimicrobial Targets
- Mechanisms of TMJ Disorders
- Perspectives on Alzheimer’s Disease
- Insights into Pedagogy in Biochemistry

Catalyst
Facets of Biochemistry & Biomedical Sciences

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Splotlight: Dr. Felicia Vulcu

Dr. Felicia Vulcu is an Assistant Professor and Undergraduate Program Advisor in the department of Biochemistry and Biomedical Sciences. Dr Vulcu was recently recognized with the President’s Award for Outstanding Contributions to Teaching and Learning. Her dedication to teaching and inspiring young students has always been outstanding. She is one of the first professors incoming Biochemistry students become familiar with and most definitely a reason for the program’s success. We asked Dr. Vulcu a few questions about how she came to this role and her perspectives on teaching and learning.

**QUESTION 1.**
What sparked your interest in teaching?
Is it what you always wanted to do?
I have always loved teaching and learning. I think both go hand in hand extremely well and I do not believe that you can teach without learning. I learn a great deal from my students. I think of teaching and learning as a two-way communication conduit and this spark came from my wonderful experience here at McMaster, both as an undergraduate and a graduate student.

**QUESTION 2.**
What would you say your general teaching philosophy is?
My teaching philosophy is an open document, constantly being molded and revised with every passing life experience. At this point in my career I no longer consider myself a teacher first and foremost. I am a learner first, a storyteller second and a teacher/facilitator third. I often reflect on my teaching philosophy and I find trends emerging, from a snapshot reflection of this in real-time versus the teaching philosophy I have amassed throughout my entire time teaching. Though the snap-shot often includes different learning elements, from the flipped-classroom to virtual labs, the overarching end goals are often the same throughout my entire teaching career. One central, tenet to my learning philosophy is safety. The safety to learn, express, create, share and experience. The other practices follow closely with engage, and reflect being the main concepts I implement in every course/curriculum I am involved in. Content, though extremely important, is not the first practice I lead with, but it is at the base of creating certain learning objectives and assessments.

**QUESTION 3.**
What has been your most rewarding teaching experience?
This is a very difficult question as I find rewarding teaching experiences every day. I am in awe of daily events in my life, and I often find myself taking a step back and reflecting on an experience. I think this is the stuff life is made of: the small, precious moments in day-to-day life that make us stop and smile. I do not believe I am answering the question, sorry about that. If I were to try and answer the question I would say my most rewarding teaching experience is a culmination of daily teaching experiences. I love my job SO MUCH and I am extremely happy to interact with such wonderful and caring students (both undergraduate and graduate) every day of my life. It is a tremendous experience.

**QUESTION 4.**
Your “active learning” approach, such as the Murder Mystery in the lab, why do you feel this is important? And what are some of the next steps you would like to take?
I feel engaging students in biochemistry is extremely important because it builds practical and transferrable skills. I often struggle with the learning objectives students come out with from a course or workshop I design. Aside from content-based learning objectives I also put a lot of emphasis on other skills, like dealing with failure (i.e. experiments not working), maintaining a positive outlook, communication, motivation, grit, perseverance and my personal favourites: curiosity and creativity. I start off all my courses by establishing a safe, nurturing environment conducive to dialogue and exchange of ideas. I then engage students in the content using various teaching practices, from project-based-learning, virtual lab simulations, case studies, puzzles, etc. Then I facilitate the inquiry process and allow students to develop ideas, create and be curious about the content. This process is truly and utterly magical when you see it in action, and I am thoroughly addicted to it. My next steps are simple: to keep engaging students in this process.

**QUESTION 5.**
How do you hope that Catalyst helps both graduate and undergraduate students?
I think Catalyst is very similar to my teaching philosophy. I hope this journal will engage students and prompt them to utilize their biochemistry content in creative ways. For undergraduate students, I see Catalyst as a medium to showcase student-led course projects and ideas. For graduate students, I see the opportunity to engage in mentorship and teamwork.

References:
Overlooked Potential of EPSP Synthase as an Antimicrobial Target

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Abstract

The rise of antibiotic resistance has necessitated a need to discover novel drug candidates. In consequence, an enzymatic protein found in Escherichia coli (E. coli) bacteria called 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) has shown promise as a novel drug target. EPSPS is an enzyme found in the shikimate pathway that forms EPSP and is essential for the downstream synthesis of vital aromatic amino acids: tryptophan, tyrosine, and phenylalanine. Using high-throughput screening (HTS), a library of 1000+ compounds was used to screen against the target of interest to discover inhibitors that significantly limited cellular growth. M9 minimal media was used in HTS to render EPSPS essential for bacteria. This media does not contain the aromatic amino acids, which would otherwise make EPSPS unnecessary for survival. Thus, it was rationalized that minimal media had to be used to avoid cell survival from external sources of amino acids. After conducting HTS and normalizing the data, 8 successful inhibitors of the enzyme were identified. However, these findings cannot say whether or not the compounds were targeting EPSPS or inhibiting cellular growth via another mechanism. Therefore, a series of secondary screens have been proposed to identify the target specificity of these 8 hits towards the shikimate pathway, EPSPS, and S3P binding site found within EPSPS, in the given order.

Introduction

The reduced susceptibility to current antibiotics available today has been a recurring issue and it has necessitated the need for discovery of novel drug candidates (1). One method of discovering novel drug candidates is to perform a screen using a library of compounds against a specific target to extend the range of targets of current antibiotics (2). Thus, the focus of this paper is to investigate an enzymatic drug target called 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase.

EPSP synthase (EPSPS) is the sixth enzyme of the shikimate pathway (Supplemental Figure 4) found in algae, higher plants, fungi, and bacteria (3). EPSPS brings its two substrates, shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP) to synthesize EPSP. The latter is essential for the synthesis of vital aromatic amino acids: tryptophan, tyrosine, and phenylalanine (3). Without these amino acids, protein synthesis is obstructed. Consequently, bacterial cells are unable to survive due to the lack of amino acids necessary for complete protein synthesis. Additionally, this pathway is not present in humans, which decreases the potential of harmful off-target binding effects during treatment (4). The combination of these factors validates EPSPS as a valuable drug target.

Currently, there is only one known inhibitor of EPSPS in the market: glyphosate. It is an effective inhibitor of EPSPS because it is a transition-state analog of PEP (Supplemental Figure 5). However, glyphosate is not used as an antibiotic due to its carcinogenic properties and its relatively low potency against bacterial cells, which is measured to be about 0.6 mg/mL for its minimum inhibitory concentration (MIC) (5, 6). This is the concentration required for glyphosate to have a potent effect which is relatively high compared to established antibiotics. Thus, the effectiveness of glyphosate as a drug is not practical due to the high amounts needed to see an effect. This is not a problem when glyphosate is used as an herbicide as concentrations are much higher than this MIC reported. Therefore, a key difference between glyphosate use on bacteria versus plants is simply the amount. Additionally, EPSPS is divided into two classes which differ in their sensitivity to glyphosate (15). Plants utilize class I EPSPS which is described as glyphosate-sensitive. However, class II EPSPS is described as glyphosate-tolerant. Pathogenic microorganisms such as Staphylococcus aureus, Staphylococcus pneumoniae, Pseudomonas sp. strain PG2982, and Agrobacterium sp. strain CP4 have all been reported to utilize class II EPSPS and consequently are resistant to glyphosate (15).

With this evidence of differences between plant-based and bacterial EPSPS, it is critical to discover novel inhibitors that are specific to class II EPSPS.

Glyphosate interacts with the PEP binding site, which is the closed conformation of the enzyme (7). This limits the type of compounds that can act as inhibitors: they would need to be relatively small to fit the closed conformation of EPSPS, and are essentially restricted to analogs of the PEP compound. Conversely, there is no known inhibitor of the other substrate binding site, S3P, which provides a foundation for novel discovery (7). Targeting the S3P binding site also targets the open conformation of EPSPS where the active site is more accessible, allowing for greater structural variability of potential
inhibitors. Without a doubt, there is much potential in focusing on the S3P binding site. Thus, high-throughput screening is an advantageous method used to search for these novel inhibitors.

Figure 1: Double-pass normalized replicate plot of HTS results for growth inhibition of E. coli K-12 (AG1) cells. E. coli K-12 (AG1) cultures were screened in duplicate for growth inhibition against a library of compounds from ChemBridge. OD measurements were taken at 600 nm following 12.5 hours of incubation. The data points were normalized using the double-pass method, and each set of replicate plates were plotted on separate axes. The blue shaded area was constructed from the hit cutoff values (3 standard deviations below the mean of each replicate data set), visualized by the blue dotted lines, and the eight data points within the shaded area represent hits that inhibit bacterial growth.

The method for discovering novel inhibitors of the S3P binding site is to begin by identifying general inhibitors of bacterial growth using high throughput screening (HTS). It is a process that takes advantage of robot automation to quickly assess the biochemical activity of thousands of compounds. Most often, these compounds are obtained from chemical libraries which provide a collection of stored chemicals both natural and synthetic (8). An important characteristic of this assay was the use of minimal media (6). Minimal media, compared to the usual lysogeny broth (LB) media used for cell cultures, contains no added nutrients except the essential components for cell growth. On the contrary, LB media provides vital amino acids to the bacterial cells, which would make EPSPS unnecessary for survival. Therefore, it was rationalized that minimal media had to be used to avoid cell survival from external sources of amino acids (6).

The data acquired from the HTS identified 8 successful hits from a compound library that significantly inhibited cellular growth. However, this does not explain whether the compounds were targeting the S3P binding site, or even EPSPS. Future works involve a series of secondary screens to improve the target specificity of these 8 hits towards the shikimate pathway, EPSPS, and S3P binding site. To verify production of EPSPS for these future assays, a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted on the E. coli K12 (AG1) cell line.

Figure 2: Characterization of purified EPSP synthase (EPSPS)-His(6) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Escherichia coli (E. coli) K-12 (AG1) cells were lysed after isopropyl-β-D-1 thiogalactopyranoside (IPTG) induction for EPSPS-His(6) protein expression. Different samples were then collected after running the cell lysate through a nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography column. Lane 1 represents the protein ladder. Lane 2 represents the cell lysate. Lane 3 is the flow-through fraction and lane 4 is the wash fraction. Lanes 5-10 represent the six elution fractions eluted using 250 mM of imidazole. The arrowhead indicates the presence of the EPSPS-is(6) protein at around 47 kDa.

Results

Z' Values show High Assay Quality

An assay plate with half positive controls and half negative controls can be set-up to calculate the Z’ factor for HTS assays. The Z’ factor is a statistical test for assessment of the assay quality. It is a dimensionless parameter used to calculate the signal separation between the highest and lowest assay readouts, which are the negative and positive controls, respectively. The negative control wells contained E. coli K12 (AG1) grown in M9 minimal media and produced the highest optical density (OD) readouts. The positive control contained E. coli grown in M9 minimal media supplemented with ampicillin, an antibiotic, and produced the lowest OD readouts. The signal separation between the negative and positive control is known as the signal...
The primary HTS sought to identify significant inhibitors of cellular growth, or hits, by screening a library of compounds against the E. coli K12 (AG1) cell line. Hits were designated as compounds presenting optical density (O.D) values below three standard deviations from the mean O.D of each replicate data set (9).

The eight hits were identified from a consolidated data set from all screens performed. Notably, data from the third screen were excluded from this set because of issues arising from the screening conditions. Firstly, the starting concentration of bacterial cells in the third screen was measured at an O.D value of 0.04, whereas the other two screens began with double the O.D. To account for this difference, the third screen was incubated for a longer period of 28 hours, which was inconsistent with the other screening periods of 12.5 hours. Moreover, the Z' value of the third screen was less than the recommended 0.5 cut-off and was relatively low compared to the other screens (9). The low Z' value indicates poor assay quality and a very narrow active window to identify potential hits, thus omitting the third screening data for the purposes of hit identification.

The data were normalized using both the single and double pass methods. A comparison of the graphs shows that single pass normalization corrected for inter-plate fluctuations that occurred due to different screening days and environments, which was necessary because not all the screens were performed on the same day. Likewise, the double pass normalization accounted for bias due to the positioning of the plates in the incubators and variations in between the wells of each plate.

**Critical Discussion of Data**

The secondary screening process requires functional and isolated EPSPS. Thus, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to verify that E. coli K12 (AG1) cell lines are successful at producing EPSPS. Supplemental Figure 6 illustrates the one letter amino acid sequence of EPSPS from the cell line of interest.

Figure 2 shows the results from the SDS-PAGE. Most notable in this gel is the presence of a thick band corresponding to the theoretical molecular weight of histidine-tagged EPSPS at 47 kDa. The elution lanes additionally contained traces of other sized proteins, notably at around 20 kDa. This band may be due to the expression of wild-type EPSPS by the bacterial strain used for protein expression, as the theoretical molecular weight of wild-type EPSPS without the hexahistidine (His(6)) tag is 20 kDa.

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process. It reduced the biases and errors that may occur during screening.

As shown in Figure 1, the eight identified compounds of interest demonstrated similarly low O.D measurements on both replicates, thereby reducing the likelihood of being false positives. Moreover, the Z’ values for these screens were both above 0.5, which indicates a strong active window and establishes the legitimacy of the identified hits.

Figure 2 shows the results from the SDS-PAGE. The thick band patterns at around 48 kDa can be explained by the presence of histidine-tags attached at the ends of EPSPS. These cells were induced using isopropyl β-D-1-thiogalactopyranoside (IPTG) to express the histidine-tagged EPSPS through a T5 promoter system found in the pCA24N plasmid in the E. coli K12 line. These histidine-tagged proteins in the cell lysate have a strong affinity for the nickel resin in the column, allowing for other cellular contents to elute in the flow-through and wash samples while most the enzyme remains in the column. The elution lanes additionally contained traces of other sized proteins. These may correspond to degraded or aggregated protein fragments of EPSPS, which will also remain in the column due to the presence of histidine-tags at the end. To troubleshoot, size exclusion column can be used to separate out the EPSPS enzymes from the other elution fractions (11).

Future Outlook

The purpose of future work is to conduct secondary screens to narrow down the hits from the primary screen to more specific targets. This firstly involves supplementation of media of the hits with the aromatic amino acids: tryptophan, phenylalanine, tyrosine. Normally, the shikimate pathway results in the generation of precursors for the aforementioned compounds (12). Thus, if there is a recovery of cell growth in the primary screen with supplementation, it will indicate that the hits are targeting a member of the shikimate pathway or an entity just downstream of the shikimate pathway.

Subsequently, the hits that target the shikimate pathway will then be used in a malachite green enzymatic assay to see if they specifically target the EPSPS in the pathway. EPSPS normally catalyzes the sixth step in the shikimate pathway, which produces EPSP and an inorganic phosphate by-product. The ammonium molybdate present in the malachite green assay should bind to free inorganic phosphate produced from the EPSPS catalyzed reaction (13). This will result in the assay solution turning green from originally being yellow. However, if the assay is exposed to hits that target EPSPS, the solution should remain yellow because no inorganic phosphate will be produced with EPSPS inhibition. This will provide information on whether EPSPS is being targeted, albeit it will not identify the active site or the conformation of EPSPS that is specifically being targeted.

Thus, leads that target EPSPS will then be subjected to further kinetic analysis to see if they target the open conformation of EPSPS, specifically at the S3P binding site. In this analysis, the hit concentrations remain constant, and S3P concentrations are altered to generate Lineweaver-Burk plots. Based on the slope and intercepts of these plots, we can determine the type of inhibition that is occurring. If the inhibitor targets the S3P binding site, the graph should display a pattern that matches competitive inhibition because it would be directly competing against S3P to bind to the S3P binding site (14). If the inhibitor is occupying the PEP binding site, the graph would display a different plot pattern that matches uncompetitive or non-competitive inhibition because the PEP binding site is an allosteric binding site with respect to the S3P binding site (14). Overall, secondary screens will allow for further identification of the target from the original primary screen.

References:


Supplementary Figures:

Supplemental Figure 1: Flowchart depicting primary, secondary, and kinetic screening process. Work to date includes a primary high throughput screen which identified 8 cellular growth inhibitors from 1240 compounds. To determine whether these 8 compounds inhibit cellular growth by inhibiting EPSPS, a secondary screen must be performed in the future. Hits from the secondary screen may then be analyzed with the use of a kinetic assay to determine whether the secondary screen hits inhibit the S3P binding site of EPSPS.

Supplemental Figure 2: Z' statistical test for ampicillin in M9 minimal media during the primary HTS. OD at 600nm was measured following approximately 28 hours of incubation for A. group E3 and 12.5 hours of incubation for B. group E1 and C. group E2. Sample number is plotted on the x-axis, and the Optical Density at 600 nm is plotted on the y-axis. Positive controls contained ampicillin at a concentration of 4 µg/mL while negative controls contain neat DMSO. The solid line through each data set represents the mean values and the dashed lines above and below the mean values represent three times the standard deviation values. The calculated Z' value is displayed near the centre of the plot.

Supplemental Figure 3: Optical Density Plots of Screening data. A. Raw optical density plot of screening data was B. single-pass rank-order normalized and then C. double-pass rank-order normalized. More than a 1000 compounds from the ChemBridge library were screened for their ability to inhibit growth of E. coli K-12 (AG1) cells in minimal media. Cells were incubated for 12.5 hours and the optical densities (O.D) of the samples were measured at 600 nm. The screen was conducted in duplicates: replicate 1 is shown in blue, replicate 2 in orange.
Supplemental Table 1: Identified Active Compounds from Primary HTS. The identified hits from the primary HTS were identified online on the ChemBridge compound database.

<table>
<thead>
<tr>
<th>Screening Well</th>
<th>Active Compounds</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>54-A02</td>
<td>2-[(4-amino-1,2,5-oxadiazol-3-yl)-1H-benzimidazol-1-yl]-N-(3-bromophenyl)acetamide</td>
<td></td>
</tr>
<tr>
<td>55-H07</td>
<td>ethyl 4-methyl-2-[(2H-1,2,4-triazino[5,6-b]indol-3-ylthio)butanoyl]amino]-1,3-thiazole-5-carboxylate</td>
<td></td>
</tr>
<tr>
<td>57-G11</td>
<td>N-1-(2,4-dimethylphenyl)-N-2-[(4-methylbenzyl)-N-2-[(4-methylphenyl)sulfonyl]glycinamide</td>
<td></td>
</tr>
<tr>
<td>59-A09</td>
<td>5-benzyl-2-[(3-bromo-4-methylbenzoyl)amino]-4-methyl-3-thiophenecarboxamide</td>
<td></td>
</tr>
<tr>
<td>64-G08</td>
<td>N-<a href="2-methoxyphenyl">(5-chloro-8-hydroxy-7-quinolyl)</a>methyl)propanamide</td>
<td></td>
</tr>
<tr>
<td>66-B02</td>
<td>N-[(4-chlorophenyl)(8-hydroxy-7-quinolyl)methyl)cyclohexanecarboxamide</td>
<td></td>
</tr>
<tr>
<td>66-H05</td>
<td>3-chloro-4-ethoxy-N-[(4-(4-methoxyphenyl)-1,3-thiazol-2-yl)amino]carbonothioyl]benzamide</td>
<td></td>
</tr>
<tr>
<td>66-H08</td>
<td>N-<a href="4-methylphenyl">(8-hydroxy-7-quinolyl)</a>methyl)cyclohexanecarboxamide</td>
<td></td>
</tr>
</tbody>
</table>

Supplemental Figure 4: Outline of the shikimate pathway and intermediates. The conversion of shikimate-3-phosphate to 5-enolpyruvylshikimate-3-phosphate in the sixth step is catalyzed by the enzyme of interest, EPSPS. Thus, inhibition of EPSPS will obstruct the shikimate pathway and ultimately prevent aromatic amino acid biosynthesis.

Supplemental Figure 5: Chemical structures of glyphosate and phosphoenolpyruvate (PEP). Glyphosate has been established as a well-known inhibitor of EPSPS as it competes with PEP for the PEP binding site. Due to its similarities in structure with PEP, glyphosate acts as a transition state analog which explains its success as a potent inhibitor. These similarities include the possession of a carboxylic acid group within both molecules. In addition, glyphosate contains a phosphonate moiety which is analogous to the phosphate group found in PEP.
Supplemental Figure 6: Complete one letter amino acid sequence for EPSPS protein. A BLAST search of the query gene was used to identify the gene sequence as that of aroA from Escherichia coli (strain K12), which codes for the protein product 3-phosphoshikimate-1-carboxyvinyltransferase (EPSPS). The image is a screen shot of the one letter amino acid sequence from UniProt.
A central role for reactive oxygen species (ROS) in the pathogenesis of temporomandibular joint disorders: All roads lead to ROS

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\textsuperscript{1}Authors contributed to equally to this work.

Abstract

Temporomandibular joint disease (TMD) is a musculoskeletal pain disorder occurring at the temporomandibular joint (TMJ), the interface of the skull's temporal bone and the mandible\textsuperscript{1}. This literature review examines the anatomy, epidemiology, biochemistry and cellular biology of TMD in order to frame underlying biochemical and cellular events within the human context of the disease. This review identifies several key elements pertaining to TMD pathogenesis, including mechanical stress-induced hypoxia-reperfusion, disruption of mitochondrial function, arachidonic acid catabolism (through prostaglandins and leukotrienes), cartilage degradation, bone resorption, and breakdown of joint lubrication. Based on a thorough analysis of established correlations and causations we propose an overarching mechanism that provides a holistic representation of TMD, something noticeably absent in the literature to-date. This mechanism clearly highlights the central role of reactive oxygen species (ROS) in the pathogenesis of TMD, a conclusion holding significant implications for both treatment and our understanding of the disease.

Introduction

The TMJ is a synovial joint located bilaterally at the jaw\textsuperscript{2}. It is directly involved in the opening and closing of the mouth, mastication, lateral excursions, and protrusion\textsuperscript{2}. Primary joint structures include the mandibular fossa, articular tubercle, and mandible head between which an articular disc is situated\textsuperscript{2}. The articular disc, composed of fibrous connective tissue, mitigates friction by separating surrounding bones\textsuperscript{2}. TMD typically arises from chronic compression of the TMJ, which induces a number of detrimental effects including the production of ROS and anterior disc displacement, a painful TMD symptom clinically known to limit jaw mobility\textsuperscript{3}.

ROS, such as superoxide anion (O\textsuperscript{2-}) and hydroxyl radicals (OH\textsuperscript{-}), serve diverse cellular functions in signal transmission, cellular activation, and regulation\textsuperscript{3}. Despite these essential roles, ROS levels must be regulated by antioxidants to prevent deleterious off-target reactivity\textsuperscript{3}. If this balance is compromised and ROS accumulate, serious consequences including DNA damage, protein denaturation and lipid peroxidation can occur\textsuperscript{3}. This phenomenon, termed oxidative stress, acts to propagate TMD\textsuperscript{3}.

Currently, few review articles discuss the effects of oxidative stress on TMD pathology\textsuperscript{3-5}. Milam et al. propose a pathway for oxidative stress-induced TMJ damage. They describe mechanical stress as the primary instigator of ROS synthesis with subsequent regulatory pathways involving ROS\textsuperscript{5}. Although recent studies continue to investigate the effects of mechanical stress on the TMJ, they fail to properly discuss the full implications of mechanical stress on the downstream ROS pathways\textsuperscript{6}. Several primary research studies have investigated specific cellular mechanisms disrupted by ROS in TMD, such as the lysing of hyaluronic acid, but rarely do they reference TMD's primary cause - mechanical stress\textsuperscript{4,6-8}. Inadequate contextual framing is also observed in a study exploring the role of iron in OH-formation via the Fenton reaction, which does not consider potential sources of iron\textsuperscript{7}. By synthesizing the limited scopes of individual papers such as these, we have created a more complete view of the role of ROS in TMD (Figure 1). Temporomandibular joint disease (TMD) is a musculoskeletal pain disorder occurring at the temporomandibular joint (TMJ), the interface of the skull's temporal bone and mandible\textsuperscript{1}. This literature review examines the anatomy, epidemiology, biochemistry and cellular biology of TMD in order to frame underlying biochemical and cellular events within the human context of the disease. This review identifies several key elements pertaining to TMD pathogenesis, including mechanical stress-induced hypoxia-reperfusion, disruption of mitochondrial function, arachidonic acid catabolism (through prostaglandins and leukotrienes), cartilage degradation, bone resorption, and breakdown of joint lubrication. Based on a thorough analysis of established correlations and causations we propose an overarching mechanism that provides a holistic representation of TMD, something noticeably absent in the literature to-date. This mechanism clearly highlights the central role of reactive oxygen species (ROS) in the pathogenesis of TMD, a conclusion holding significant implications for both treatment and our understanding of the disease.
ROS have expansive involvement in the pathogenesis of TMD\textsuperscript{8-18}. One ROS-producing mechanism is the xanthine oxidase pathway (Figure 1).\textsuperscript{9-10} First, some form of mechanical stress at the TMJ, such as tight clenching or prolonged maximal mouth opening during a dental procedure, increases intra-articular pressure\textsuperscript{9}. Once joint pressure exceeds 40 mmHg, peripheral arteriolar blood pressure is overpowered, resulting in transient hypoxia of TMJ tissues\textsuperscript{9}. Hypoxia induces a metabolic shift in affected tissues that produces ROS following TMJ relaxation and tissue reperfusion.\textsuperscript{14-15} This shift is mediated by xanthine oxidase, which generates $O_2^-$ during reoxygenation by reacting in the presence of oxygen with hypoxanthine that accumulates in the hypoxic state.\textsuperscript{8, 14-18}

**Xanthine Oxidase-Mediated ROS generation**

ROS are produced during cell activation, stress, and tissue injury. Xanthine oxidase is a key enzyme involved in the production of ROS. It catalyzes the conversion of hypoxanthine to xanthine and $O_2^-$, which can then react with oxygen to form superoxide ($O_2^-\cdot$). This process is particularly important in the context of TMD, as it can lead to oxidative stress and tissue damage.

**Mitochondria**

Mitochondria represent another source of ROS. Under physiological conditions, mitochondria release $O_2^-$; this release is exacerbated under hypoxic conditions due to mitochondrial inhibition, as follows and shown in Figure 1. Generally, mitochondria attempt to pass off electrons through the mitochondrial respiratory chain to synthesize water. Oxygen depletion stymies this pathway, causing electron leakage. This leakage engenders $O_2^-$, which leads to the oxidative decarboxylation of $\alpha$-ketoglutarate and the subsequent inhibition of its cofactor, prolyl hydroxylase domain-2 (PHD2).\textsuperscript{11} The role of PHD2 is to hydroxylate hypoxia-inducible factor 1$\alpha$ (HIF-1$\alpha$) in order to target it for proteasomal degradation. Therefore, its inhibition causes HIF-1$\alpha$ accumulation.\textsuperscript{10, 11, 19} Amassed HIF-1$\alpha$ acts as a subunit to complete the HIF-1 transcription factor.\textsuperscript{10, 11, 19} Increased HIF-1 leads to certain detrimental effects implicated in TMD, as discussed in 'Cartilage degradation and bone resorption'.

**Arachidonic Acid Catabolism: Prostaglandins & Leukotrienes**

Prostaglandins (PGs) are first synthesized from arachidonic acid (AA) by most human cells.\textsuperscript{20} In the context of TMD, PGs increase ROS synthesis. Mechanical stress, cytokines, and growth factors can release AA from the phospholipid membrane. ROS stimulate cytokine production, causing upregulation of PG synthesis. PG synthesis occurs via the enzyme cyclooxygenase-2 (COX-2), which produces ROS as a byproduct (Figure 1). This results in greater levels of ROS in the joint.\textsuperscript{22}

Once released, AA is metabolized by COX-1 and COX-2 to form PGH\textsubscript{2}. PGH\textsubscript{2} is subsequently converted into PGE\textsubscript{2}, a process known to generate ROS as a byproduct.\textsuperscript{23} In fact, Kerins et al. demonstrated a reduction in orofacial pain in a rat TMD model following COX-2 inhibition by measuring...
meatime patterns. Untreated rats had longer mealtimes (indicating more pain) than treated rats, suggesting COX-2 is integral to TMJ inflammation and pain. Further studies showed COX-2 inhibition downregulates NADPH activity, thus reducing ROS levels. This is potentially because AA can synthesize ROS through NADPH independent of COX-1 and COX-2. An alternative to conversion into PGs, Cocco et al. demonstrated that AA produces ROS by interfering with the mitochondrial electron transport chain, as AA addition to respiring cell increased hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) levels compared to basal conditions.

Leukotrienes (LTs) may also stimulate inflammation and subsequent ROS production in the TMJ. LT synthesis begins when 5-lipoxygenase-activating protein translocates AA to 5-lipoxygenase, which converts the AA to LTA\textsubscript{4}. LTA\textsubscript{4} then undergoes one of several conversion pathways, the most pertinent of which forms LTB\textsubscript{4}. LTB\textsubscript{4} produces ROS through a rac-dependent pathway which mediates AA-induced ROS generation through a PLA\textsubscript{2} linked cascade. Woo et al. explored LTB\textsubscript{4} as a crucial mediator in tumour necrosis factor-\alpha (TNF-\alpha) induced ROS generation via NADPH. Results revealed dose-dependent ROS upregulation by LTB\textsubscript{4}, the effects of which were inhibited by an LTB\textsubscript{4} receptor (BLT) antagonist. Furthermore, blocking of the specific G-protein coupled to BLT receptors decreased ROS production in a dose-dependent manner. Hence, these studies thus delineate the vital role of PGs and LTs on the production of ROS through arachidonic acid catabolism.

Once ROS accumulate via the aforementioned pathways, including the xanthine oxidase, mitochondria pathway, and arachidonic acid pathways, they damage collagen and the lubricative layer by reacting with key molecules in the joint and compromising their functional integrity through processes which will forthwith be explored.

**Cartilage degradation and bone resorption**

The TMJ is composed of bone, a fibrous articular disc, and fibrocartilage (Figure 1), which contains fibroblasts, chondrocytes, and type I and II collagen. Cartilage plays an essential role in TMJ function by reducing joint loading and providing a surface for the articulation of the disc. ROS indirectly degrade the type I collagen found in the articular cartilage, as discussed in detail below.

As explained previously, hypoxia induces ROS and subsequent HIF-1\alpha accumulation by stabilizing the HIF-1\alpha subunit. HIF-1 binds the hypoxia response element on the gene promoter of vascular endothelial growth factor (VEGF), consequently upregulating VEGF expression under hypoxic conditions (Figure 1). Experiments in cardiac myocytes demonstrate direct HIF-1\alpha stabilization by mechanical stress, reinforcing the critical role ROS generation plays in TMD. Pufe et al. used bovine cartilage discs to imply that HIF-1\alpha induced by mechanical stress increased VEGF expression.

ROS-induced VEGF upregulation causes multiple degenerative consequences contributing to TMD such as cartilage degradation and bone resorption (Figure 1). Matrix metalloproteinases (MMPs), specifically MMP-1 and MMP-13, are known to degrade fibrocartilage collagen, which forms the cartilaginous extracellular matrix that is essential to the health of the TMJ.

Pufe et al. observed upregulation of MMP-1, MMP-3, and MMP-13 and downregulation of tissue inhibitors of matrix metalloproteinases-1 (TIMP-1) and TIMP-2 in articular discs following VEGF induction by mechanical overload. Similarly, another experiment observed increased MMP-1, MMP-3, MMP-9 and TIMP-1 mRNA levels in chondrocytes subjected to mechanical stress. Further experiments ratified these results of MMP mRNA upregulation following tensile stress, illustrating how ROS can affect several downstream proteins that act detrimentally to TMJ integrity.

As previously described in this article, mechanical stress can produce ROS in the TMJ. Regarding condylar bone resorption, Tanaka et al. observed increased VEGF immunopositive chondrocytes following mechanical stress in rat TMJs. Furthermore, the area subjacent to the deep hypertrophic cartilage demonstrated signs of significant increase in osteoclast presence. VEGF chemo-attracts osteoclasts; inhibition of VEGF function via antagonist chimeric proteins or anti-VEGF antibodies impairs osteoclast invasion. Thus, the stimulation, differentiation, and migration of osteoclasts into cartilage are all induced by VEGF production. This may result in cartilage destruction through vascular invasion, which converts cartilage into bone. Therefore, the production of ROS that leads to VEGF accumulation causes physiological degradation of the TMJ.

**TMJ lubrication, hyaluronic acid, and oxidative stress**

The lubricative layer expedites the articulation of the various bony components of the TMJ and is thus essential to normal function. Surface-active phospholipids (SAPLs) are the central molecules responsible for this phenomenon. Their polar heads bind the articular surfaces while their dual hydrophobic moieties extend into the joint space forming hydrogen bonds. The resulting hydrophobic interface enables low friction coefficients even under high loads. Hills observed that washing away SAPLs and lamellar body depositions caused a 150% friction increase, confirming the importance of SAPLs in TMJ lubrication.

Hyaluronic acid (HA) is widely accepted to be protective in joint lubrication. HA was initially presumed to directly lubricate the TMJ, but this hypothesis was overturned when hyaluronidases were observed to negligibly affect joint lubrication. It was later established that polymeric HA fulfills a protective, body-guarding function for SAPLs through...
dose-dependent inhibition of phospholipase A2 (PLA2). PLA2 degrades SAPLs and is constitutively secreted into the TMJ synovial fluid, making HA's bodyguarding function critical. Several nuances can be applied to this general understanding of HA function. Firstly, HA can be subdivided into high-molecular weight (HMW) and low-molecular weight (LMW) varieties which are produced by three separate HA synthases (HAS)52-67. HAS-1 and HAS-2 polymerize HMW-HA at ~2000 kilodaltons (kDa) and are upregulated by pro-inflammatory cytokines and transforming growth factor β-1 (TGFβ-1)56,57. HAS-3 polymerizes LMW-HA at only 200 kDa and is upregulated by pro-inflammatory cytokines but downregulated by TGFβ-153-57. HMW-HA scavenges ROS at the expense of molecular integrity more effectively and broadly than LMW-HA68. HMW-HA also inhibits PLA2 activity, whereas LMW-HA exacerbates it53,58-60. Wang et al. examined the effects of HMW-HA on the gene expression of 16 osteoarthritis-associated factors in fibroblast-like synoviocytes (FLS) from human osteoarthritic knees61. They found HMW-HA significantly downregulates interleukin-8, inducible nitric-oxide synthase, aggrecanase-2 and TNF-α mRNA61. Down-regulatory effects against all 16 tested factors were observed, yet insufficient sample size (15) hindered the significance of these results61. Wang et al. further demonstrated that inhibition of CD44 (HA’s major receptor) with monoclonal antibodies impeded these down- regulatory effects, invoking CD44 as an intermediate in HMW-HA’s gene regulatory effects (Figure 1)61.

HA filaments aggregate non-covalently with the proteoglycans aggrecan and lubricin, this interaction is facilitated by the glycoprotein link protein (LP)1,46,48,60,62-63. Aggrecan is a macromolecule (~1-4MDa) which contributes to joint loading capacity, binding both HA and LP63. Lubricin acts as a water-soluble carrier of SAPLs to enable their deposition on articular surfaces1,46. Finally, LP associates with HA and both proteoglycans through separate binding domains to facilitate aggregation60,63.

ROS interrupt this tightly-knit system in diverse ways1,3,58,60,63,64. For example, Roberts et al. found OH- mediates LP peptide bond cleavage60. They postulate OH- radicals are produced from H2O2 by a Fenton reaction localized at a free iron ion chelated by histidine residues within LP itself60. This hypothesis represents a useful microcosm illustrating how free transition metal ions (like iron) can exacerbate the negative effects of ROS. Importantly, HA suffers limited cleavage by H2O2, but more vigorous depolymerisation via OH- attack58,60,63. These destructive effects compromise protein integrity and cumulatively abolish all aforementioned intermolecular associations (save for that between HA and LP), thereby exposing the SAPLs to PLA2-mediated lysis1,3,49. Loss of SAPLs results in a thinner lubricative layer, increasing joint friction63.

Increased friction can stress joint tendons, culminating in progressive anterior disc displacement1,3,60,63.

Cumulatively, these studies demonstrate a significant protective, anti-inflammatory role for HMW-HA in the TMJ. This is supported by in-vivo animal studies, such as Lemos et al.’s study of osteoarthritic rat RMJs that found a decrease in adverse morphological changes and MMP activity following HMW-HA administration65. Manfredini et al. reviewed HMW-HA injection in humans and found a consistent and lasting reduction in pain66. Despite these promising results and a clear biochemical basis, HMW-HA therapy must be thoroughly compared to corticosteroids in future trials to elucidate the superior treatment66.

Conclusion

ROS are central to the pathogenesis of TMD, yet very few articles holistically discuss the effects of oxidative stress on the TMJ1,3,18. To our knowledge, no review article has aimed to create a single picture broadly summarizing all molecular pathways involved in TMD- pathogenesis. Following a comprehensive review of the literature, we found that mechanical stress- induced hypoxia-reperfusion disrupts the respiratory chain reaction of mitochondria, contributing to ROS generation10,12. AA also plays a critical role in ROS production through the metabolism of AA into PGE2 by COX enzymes20-21,23. Furthermore, AA is an important leukotriene precursor30. Leukotrienes such as LTβ4 are crucial mediators in TNF-α-induced, AA-induced, and rac- dependent ROS generation pathways3,55. The upregulated ROS production can increase HIF-1 and VEGF. VEGF’s subsequent upregulation of vascular invasion, and MMP and osteoclast activity can degrade the cartilaginous components of the TMJ, thus compromising its structural integrity. As ROS accumulate in the TMJ they degrade HA, exposing SAPLs to PLA2- mediated lysis. This thins the lubricative layer, increasing joint friction and thereby engendering anterior disc displacement1,3,58,60,62-64. Based on information presented in this review it is not surprising that HMW-HA has shown therapeutic potential in both animals and humans65-66. Indeed, this review supports a central role for ROS in TMD pathogenesis as observed in Figure 1, revealing its importance as a target for TMD treatments.

References:


Alzheimer’s disease: have we been focusing on the wrong proteins?

Ryan Gotesman

Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder and the most common form of dementia, characterized by neuronal atrophy in the cerebral cortex and hippocampus\textsuperscript{1}. The disease commonly affects those over the age of 65, clinically manifesting as loss of memory and cognitive decline\textsuperscript{2}. The neuropathological origins of AD have been classically attributed to misfolded proteins, specifically plaques composed of the amyloid-β (Aβ) peptide and neurofibrillary tangles (NFTs) consisting of aggregates of hyper-phosphorylated tau. Currently no disease-modifying drugs capable of stopping or slowing the progression of the disease exist and only symptomatic treatments are available. As 1 in 85 people worldwide are projected to suffer from AD by 2050, an improved understanding of the disease and the development of novel therapies will become more pressing with each passing year\textsuperscript{3}.

Amyloid-β and Tau

Aβ is formed from the cleavage of amyloid precursor protein by beta-site amyloid precursor protein cleaving enzyme 1 (BACE1), resulting in peptides of about 40 residues in length\textsuperscript{4}. Though most Aβ peptides are harmless Aβ42, an amyloid-β peptide with 42 residues, has the tendency to misfold forming fibrils which can aggregate and develop into plaques\textsuperscript{5}. Although the physiological role of Aβ is unclear, the misfolded peptide can induce the formation of β-sheet rich neurotoxic tau oligomers\textsuperscript{6}. Tau is a member of the microtubule associated protein class of proteins, and is responsible for stabilizing microtubules and promoting their polymerization in neurons\textsuperscript{7}. In AD brains, tau becomes hyper-phosphorylated, self-aggregating into tightly packed filaments and disrupting microtubule structure.

The last 20 years of AD research have focused on developing therapies targeting Aβ with little success. Particularly the failure of Aβ antibodies, capable of reducing plaque density in the brain, as well as Aβ vaccinations in improving cognition or functional ability, coupled with the existence of cognitively healthy individuals with abnormally high levels of Aβ has called into question the soundness of the “amyloid hypothesis”\textsuperscript{8-11}. Focus is slowly shifting to tau as the target of choice in treating AD, although two recent trials aimed at reducing tau hyper-phosphorylation ended in failure\textsuperscript{12,13}. However, a growing body of evidence is suggesting that the scientific community has been focusing on the wrong proteins and that improved understanding of insulin, rather than Aβ or tau, may hold the key to treating AD.

Link between Alzheimer’s disease and Insulin Resistance

The human insulin protein is comprised of 51 amino acids and is secreted from pancreatic β cells into the blood stream in response to high levels of plasma glucose\textsuperscript{14}. Through binding with the insulin receptor, insulin can promote cellular uptake of glucose from the blood. Insulin resistance is a hallmark of type 2 diabetes (T2D) and occurs when cells are unresponsive to insulin and fail to absorb glucose. Chronic insulin resistance can lead to renal and cardiovascular disease as well as blindness\textsuperscript{15,16}. Interestingly, in AD, insulin resistance is also observed in the body and brain, with patients commonly possessing high fasting plasma glucose levels, hyperinsulinemia, and impaired glucose tolerance\textsuperscript{17}. Indeed, a quick glance at an FDG-PET scan reveals glucose metabolism is reduced throughout much of the brain in those with AD\textsuperscript{18}.

There is a great deal of epidemiological evidence linking AD and the insulin resistance commonly observed in diabetes. In the Rotterdam cohort study, where over 6000 individuals were followed for as many as 6 years, diabetes was found to double the risk of developing dementia\textsuperscript{19}. Recent work by Xu et al. confirmed these findings and, in their sample, risk of developing AD or vascular dementia increased three times in those with diabetes\textsuperscript{20}. As insulin stimulates glucose absorption in the brain, it is possible that insulin resistance may lead to neuronal energy imbalance and oxidative stress, causing the accumulation of misfolded proteins commonly implicated in AD pathogenesis. Supporting this claim, mouse studies have shown disruption of pancreatic insulin secretion or neuronal insulin receptor expression can lead to formation of plaques and tangles as well as cognitive decline\textsuperscript{21,22}.

Viewing Alzheimer’s disease through the lens of Insulin Resistance

Elucidating the link between AD and insulin resistance may help shed light on the processes that culminate in the formation of plaques and tangles. One of the causes of amyloid plaque accumulation may be an imbalance in the clearance and production of Aβ. Interestingly, a recent study on rats demonstrated that chemically stimulated insulin deficiency leads to increased expression of BACE1, the enzyme involved in the breakdown of amyloid precursor protein to Aβ\textsuperscript{23}. This, coupled with the fact that insulin can suppress expression of amyloid precursor protein, suggests how insulin resistance
could push the equilibrium in favour of Aβ production24. Insulin also promotes release of Aβ from in vitro cell cultures and accelerates trafficking of the protein from the Golgi apparatus to the plasma membrane25. Hence, insulin resistance may lead to more Aβ remaining within the cell where it can potentially form into plaques.

Insulin resistance may also serve to explain the underlying tau pathology of AD. One of the mechanisms by which tau hyper-phosphorylation can occur is through over-activity of its many kinases. One main tau kinase is glycogen synthase kinase 3 (GSK3) and overexpression of GSK3 in mouse brains was found to induce hyper-phosphorylation of tau26. Normally, the binding of insulin to its receptor leads to the recruitment of phosphatidylinositol 3-kinase (PI3K) which activates a number of downstream effectors ultimately leading to GSK3's phosphorylation27. As phosphorylation of GSK3 induces a conformational change that masks the protein’s active site, in this way insulin can inhibit GSK3 activity28. Insulin resistance will therefore lead to increased levels of GSK3 activity, stimulating hyper-phosphorylation of tau and partially explaining the NFTs commonly observed in AD.

**Insulin-Related AD Therapies**

Several clinical trials and cohort studies have shown therapies targeting the insulin resistance component of AD can have significant efficacy. One early trial with twenty five participants found daily intranasal insulin treatment allowed greater retention of verbal information and improved attention in patients with early AD29. A larger, more recent trial with 104 individuals found AD participants given intranasal insulin experienced improvements in memory and functional ability30. Importantly, no adverse events were reported for the insulin therapy and larger and longer studies investigating insulin as a therapeutic for AD must be conducted.

Apart from insulin, another potential therapy for AD is metformin, the most commonly used drug to treat insulin resistance in diabetes. Unfortunately, few if any randomized control trials investigating metformin’s impact on AD have been conducted and observational studies must be used to gauge metformin’s potentially beneficial effects. In a large cohort study of approximately 150000 individuals with T2D from Taiwan, taking metformin was found to reduce risk of dementia by approximately 35% in 8 years31. In contrast to these findings, a large case-control study from the United Kingdom found that individuals were at slightly higher risk of developing AD if they received metformin in the long-term32. Clearly, more research must be done to investigate metformin’s clinically efficacy in treating AD.

**Conclusion**

A growing body of evidence is suggesting we begin to view AD, not as a protein misfolding disorder, but rather as a metabolic one. If AD truly does stem from insulin resistance, and it is insulin resistance that ultimately stimulates the formation of misfolded amyloid and tau, the failure of therapies targeting these proteins could be easily explained as the chief cause of AD is not being addressed. Interestingly, of the few drugs actually approved to treat AD, none of them even interact with amyloid-β so it is curious that the scientific community continues to pursue amyloid-β centered therapies with such constricted vision. Clearly a new approach to combating AD is required and a deeper understanding of insulin’s role in protein misfolding pathways will help guide future efforts to slow and reverse the progression of AD. Hopefully in the coming years the relationship between insulin resistance and AD will be more readily recognized by clinicians and researchers and pursued with as much fervour as the amyloid hypothesis.

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